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SECTION MEETINGS

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Lack of Effect of Disodium Calcium EDTA on Human Serum Cholesterol and Low-Density Lipoproteins. (24957)

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Conflicting results have been reported of the effect of ethylenediamine tetraacetate (EDTA) upon dietary induced hypercholesterolemia in experimental animals(1-3). In man a reduction of serum cholesterol was noted following the use of EDTA when administered intravenously(4), and subsequently oral effectiveness of the drug was claimed(5). In the latter instance, however, the simultaneous use of a lowered animal fat intake made it difficult to evaluate the contribution of the chelating agent. More recently a marked decrease in beta lipoproteins, determined by paper electrophoresis, was found in 5 psoriatic patients after 10 days of oral disodium calcium EDTA therapy in doses of 1.5-3 grams daily(6). Accordingly the present study was undertaken to evaluate the effect of the drug upon serum cholesterol and low-density lipoproteins in man.

Materials and methods. Eleven patients were studied. The series included individuals of both sexes, in different age groups, and had an approximately equal number of healthy subjects and those with atherosclerotic cardiovascular disease. There was no dietary change during the study. Three grams of EDTA[†] were prescribed daily, in divided doses for 4 weeks. Blood samples were drawn for serum cholesterol and low density lipoprotein determinations prior to and at the end of the 4 week medication period. Cholesterol was measured by the method of Kingsley and Schaffert(7), and the lipoproteins were ultracentrifugally analyzed.

Results. The results are shown in the Ta-

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[*] Supported by grant from Riker Labs.

[†] Versenate—Supplied by Riker Labs.

TABLE I. Serum Cholesterol and Low-Density Lipoprotein Values before and after 4 Weeks Therapy with Versenate in 11 Patients.

				Low-density lipoproteins*					
		Cholesterol		Sf 0-12		Sf 12-400		A.I.†	
Age	Sex	Control	V'senate	Control	V'senate	Control	V'senate	Control	V'senate
58	♀	1012	1065	1407	1259	310	331	195	184
57	♀	270	299	438	541	180	125	75	74
66	♂	210	187	296	339	405	221	101	73
65	♀	240	302	456	517	225	193	85	86
68	♀	302	532	673	719	518	564	158	171
70	♀	299	302	526	517	406	437	124	128
72	♂	272	265	544	521	355	284	117	102
54	♂	253	233	485	493	210	196	85	84
29	♂	225	249	396	434	409	451	111	122
40	♂	493	651	920	1111	252	281	136	160
47	♂	325	302	639	634	283	322	113	120

* Ultracentrifugally analyzed at Inst. of Medical Physics, Belmont, Calif.

† Atherogenic Index $= \dfrac{\text{Sf 0-12 lipoproteins} + \text{Sf 12-400} \times 1.75}{10}$.

ble. There were no consistent changes in any of the lipid categories beyond those which may have occurred spontaneously.

Discussion. The findings of the present study showed no cholesterol or lipoprotein lowering effect of the medication. This implies that the results previously reported(5) were probably due to the lowered fat content of the diet rather than the chelating agent.

Summary. Calcium disodium EDTA, in total dose of 3 g daily for 4 weeks, had no effect upon serum cholesterol and low-density lipoproteins of 11 humans.

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In vitro Assay of Hog Intrinsic Factor Concentrates Employing Paper Electrophoresis and Co^{60}-B_{12}.* (24958)

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Almost thirty years ago the substance in normal gastric juice, known as intrinsic factor, was discussed by Castle(1). Many attempts have since been made to isolate the active principle in pure form. Progress has been made, although electrophoretic and chromatographic evidence suggests that even the very best preparation is only 10-20% pure. Isolation and purification work on intrinsic factor has been hampered by lack of adequate assay procedures. At first the only method available was based on extent of hematological response registered by a pernicious anemia patient in relapse(2). This is still the only procedure accepted by the USP Anti-Anemia Board for evaluating potency of commercial intrinsic factor concentrates (IFC). Recently assay methods have been proposed suitable for use with pernicious anemia patients in remission. These methods are based on increase of absorption of radioactive Vit. B_{12} brought about by ingestion of intrinsic factor. The procedures make use of hepatic uptake(3), fecal excretion(4), and urinary excretion data (5). The urinary excretion method of Schilling(5) has been used extensively and represents a real step forward. This method, however, suffers all disadvantages of a clinical assay and has all errors inherent in any biological procedure. Several attempts to develop an *in vitro* assay for hog IFC have been reported based on total Vit. B_{12} binding capacity(6). All these attempts have failed, because of presence of multiple binders in even the purest IFC measured. Hoff-Jorgensen(7) reports success in standardizing commercial intrinsic factor preparations employing a strain of *E. coli* isolated from feces of a pernicious anemia patient. Miller(8) used the stimulating effect of IFC on absorption of radioactive Vit. B_{12} by rat liver slices as a measure of activity. Herbert(9) extended this work to provide for sequential incubation of rat liver slices in intrinsic factor—containing buffer, followed by Co^{60}-B_{12}. Minard and Wagner(10) found that uptake of Co^{60}-B_{12} by rat liver homogenate can be used as *in vitro* assay of hog intrinsic factor of pyloric origin. There is still need for an assay of intrinsic factor activity which does not require a biological environment. This assay should apply to all samples possessing clinical activity, regardless of origin and method of preparation. In the present study attention was centered on Vit. B_{12} binding components present in hog IFC, since all evidence available suggests binding as a necessary, although not sufficient, condition of activity. Paper electrophoresis is used to separate various binders in the presence of sufficient radioactive Vit. B_{12} to saturate all binding sites. Tagging of Vit. B_{12} permits ready location and quantitation of the binders. A correlation of one particular binder with activity thus provides the basis for an *in vitro* assay of intrinsic factor activity based on physical data alone.

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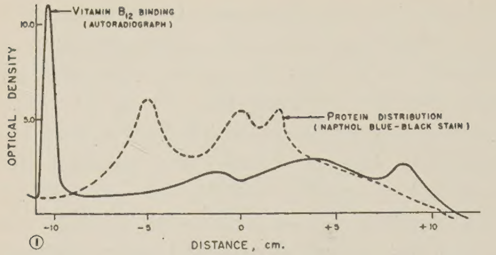
* Presented in part before Fed. of Am. Soc. for Exp. Biol., Philadelphia, Apr. 15, 1958.

Methods. Electrophoretic assay. The test solution was made in 6.2 phosphate buffer at ionic strength 0.05. Concentration of intrinsic factor was 40 mg/ml; concentration of radioactive vitamin was 10 μg/ml. The samples ranged in activity from 2 mg to 100 mg. The tagged vitamin was paraben free and had a specific activity of 0.97 $\mu c/\mu g$. Electrophoretic separation of B_{12} binders was carried out in horizontal paper electrophoresis unit (Reco), employing strips of Whatman No. 1, 50 cm x 2.5 cm. A volume of 25 λ of test solution was applied as a thin line 35 cm from the anode. Separations were carried out for 24 hours at a potential of 350 volts. After electrophoresis, the strips were air dried and autoradiographed, according to conditions outlined below. The processed films were analyzed in recording densitometer (Spinco Analytrol equipped with a B-2 cam.) Where data on protein distribution was desired, the electrophoretic strips were stained with napthol blue black and read in the densitometer. The autoradiographic procedure called for contact between strip and Kodak No-Screen X-ray film (1⅞" x 16") for 17 hours. The films were developed using standard technic. An internal standard of radioactive B_{12} was run with each film. All areas were corrected for variations in film exposure and processing conditions as revealed by response of the standard.

Results. Protein distribution data. In our early studies data were collected comparing distribution of proteins with distribution of Vit. B_{12} binders. A sample active clinically at 5 mg/day gave results shown in Fig. 1. It will be seen that major protein components are not the same as the major B_{12} binding components. This explained why our previous efforts to correlate clinical activity with Tiselius electrophoretic data had failed.

Correlation of Vit. B_{12} binding with clinical activity. Nine samples of hog IFC ranging in clinical activity from 1.5 mg to 75 mg were used. These samples were clinically assayed at 3 levels by a modified Schilling technic(11) to make certain that the activity level of each sample was established beyond doubt. The clinical data are given in Table I. Data on distribution of B_{12} binders in each of these samples were obtained following standard



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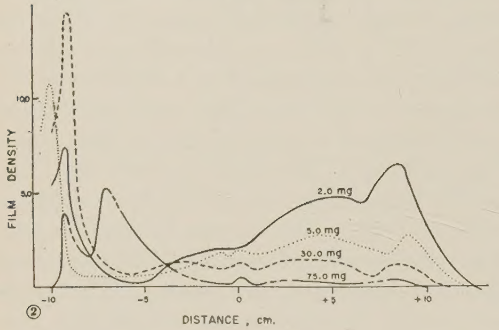

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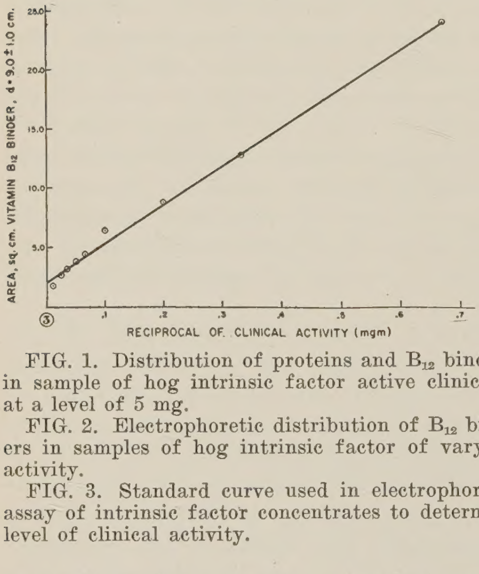

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FIG. 1. Distribution of proteins and B_{12} binders in sample of hog intrinsic factor active clinically at a level of 5 mg.

FIG. 2. Electrophoretic distribution of B_{12} binders in samples of hog intrinsic factor of varying activity.

FIG. 3. Standard curve used in electrophoretic assay of intrinsic factor concentrates to determine level of clinical activity.

autoradiographic procedure. Typical curves are shown in Fig. 2. It was noted that all samples contained more than one B_{12} binder, but more important, that they all contained a B_{12} binder that moved 9 ± 1 cm. If this figure is corrected for electroosmotic flow, the distance is 18 cm. This gives an electrophoretic mobility on paper of -3×10^{-5}cm^2/sec. volt. For convenience and since standard conditions are used, the uncorrected figure of 9 cm is preferred. A check showed that the amount of this binder appeared to correlate with clinical activity of the sample. The binding data are given in Table I. When the area of the peak at 9 ± 1 cm, a measure of B_{12} bound by that particular component, is plotted against reciprocal of the clinical activity in mg, a straight line is obtained, Fig. 3. Since samples involved in this study, all of hog mucosa origin, had been treated in a wide variety of ways and had been exposed to various types of environment, this finding of a linear relationship is meaningful.

Use of standard curve on unknowns. Using Fig. 3 as our standard curve, the work was extended to a large number of unknown concentrate samples which had never been assayed clinically. From electrophoretic assay data activity levels were predicted. Each sample was then assayed clinically by the Schilling technic at the predicted level. A few typical results are shown in Table II. It will be noted that all samples were fully active at the predicted levels. Close to 100 unknown hog IFC samples have been run so far ranging in activity from 2 mg to 100 mg. In every instance agreement between electrophoretic assay and Schilling data is excellent

Discussion. In arriving at final conditions of the method, the electrophoretic conditions of paper, pH, ionic strength and time have all been explored to make certain that maximum resolution of the B_{12} binders is obtained. A dialysis step was not included in our standard assay procedure, since all of the concentrates used to establish the assay had been dialyzed at least once during their preparation. Average specific conductivity of our test solutions (4%) was 5.0×10^{-3} mhos. Samples which have not been freed of highly conducting materials may need to be dialyzed before they are assayed.

In the autoradiographic step every effort has been made to reduce film exposure time. This includes a 20-fold concentration of the commercial solution of tagged vitamin. When B_{12} of higher specific activity becomes available, the exposure time can be reduced further. The present overnight regimen, however, is quite convenient.

In place of employing autoradiography, standard counting procedures can be used directly on electrophoretic paper strips. Counting can be carried out by scanning 1 cm segments. The disadvantage of this method is the discontinuous nature of the count data, making it difficult to quantitate amounts of

TABLE I. Clinical and Electrophoretic Data on Standard IFC Samples.

			Clinical data (Schilling) B_{12} Co^{60} recovered in urine, %			Electrophoretic data		
Standard sample	Patient	Level, mg	Base	Standard	Sample	Activity level, mg	Distance, cm	Area, cm^2
1	R.T.	3.0	0.7	6.5	8.7	1.5	8.8	24.0
	I.W.	1.5	0.2	7.0	7.5			
	C.C.	1.5	0.7	6.0	7.7			
	E.M.	0.75	1.0	6.8	3.4			
2	A.H.	6.	2.1	6.0	8.6	3.	8.4	12.8
	C.C.	3.	0.7	6.0	6.2			
	E.M.	3.	1.0	6.8	6.1			
	I.W.	1.5	0.2	7.0	3.8			
3	B.H.	10.	0.0	12.2	22.7	5.	9.3	8.8
	A.S.	5.	3.2	8.9	9.7			
	B.H.	5.	0.0	12.2	12.0			
	F.J.	2.5	1.5	15.5	7.8			
4	C.C.	20.	0.7	6.0	7.5	10.	8.3	6.4
	L.D.	10.	3.5	18.3	15.0			
	C.C.	10.	0.7	6.0	7.0			
	A.K.	5.	1.0	10.4	5.8			
5	E.P.	30.	1.3	7.0	10.0	15.	9.0	4.4
	C.R.	15.	1.5	15.2	14.1			
	J.P.	15.	0.8	9.6	10.0			
	E.G.	7.5	7.3	27.2	11.5			
6	B.G.	40.	1.0	11.8	17.5	20.	8.2	3.8
	J.P.	20.	0.8	9.7	8.8			
	F.C.	20.	1.8	6.8	6.9			
	R.K.	10.	1.5	6.6	3.9			
7	J.P.	60.	0.8	9.7	8.0	30.	8.4	3.2
	J.F.	30.	0.0	14.0	10.9			
	L.D.	30.	3.7	18.3	14.2			
	C.J.	15.	2.7	16.7	4.6			
8	P.J.	80.	1.7	15.5	20.6	40.	8.5	2.6
	P.J.	40.	1.7	15.5	15.5			
	R.B.	40.	1.0	11.8	11.8			
	R.B.	20.	1.0	11.8	6.0			
9	R.K.	150.	1.5	6.6	16.5	75.	8.4	1.8
	J.P.	75.	0.8	9.7	14.0			
	F.C.	75.	1.8	6.8	6.9			
	F.W.	37.5	2.0	18.0	8.0			

TABLE II. Application of Electrophoretic Assay to Unknown Hog Intrinsic Factor Concentrates.

Electrophoretic assay				Clinical assay (Schilling) B_{12} Co^{60} recovered in urine, %*			
Sample	Distance, cm	Area, cm^2	Predicted level, mg	Patient	Base	Standard	Sample
26	9.5	15.0	3.	S.C.	3.3	8.4	7.7
C-10	8.5	6.0	10.	P.U.	5.5	17.1	16.5
27	8.3	4.1	15.	H.S.	2.5	14.2	16.0
17	8.3	2.8	40.	L.H.	.5	9.2	10.8
7221	8.6	4.2	15.	R.B.	1.0	7.5	9.3
46	9.3	11.0	4.	B.H.	.7	9.2	8.0
ED #3	8.7	9.3	5.	A.W.	2.1	6.0	8.6
45B	9.5	17.0	2.5	R.T.	.6	5.0	5.7
42	8.4	3.5	25.	W.W.	3.3	9.8	13.5
7223	8.8	3.1	30.	G.E.	1.4	8.4	8.6

* All clinical assays were obtained at the predicted levels.

the various binders. Use of a radiochromatogram scanner would overcome this objection.

The assay calls for use of excess of Vit. B_{12} sufficient to satisfy all binding sites and to permit a check for presence of unbound B_{12} on the autoradiograph. When substantially lesser amounts of B_{12} are used, no free B_{12} is seen, indicating that the binding equilibria favor strongly complex formation. No evidence suggesting preferential binding of Vit. B_{12} by any of the binders present has been observed.

Since the standard curve relating peak area and clinical activity is a straight line, its equation can be readily determined. This line is, however, very much influenced by the particular electrophoretic and autoradiographic conditions employed and by the choice of clinical assay procedures. A standard curve needs to be worked out for each laboratory using the assay. All unknown samples evaluated by the electrophoretic assay and checked clinically have been of hog origin. Some originated in the pylorus; others came from the duodenum. Extension of the assay to human gastric juice is now under study.

The correlative data presented do not include any inactive samples. A number of such samples, however, have been run, and in every instance the lack of any binder in the 9 cm. area has coincided with lack of any clinical response at high levels. In this work many active samples have been inactivated in the laboratory under various conditions of pH and temperature. The stability pattern for intrinsic factor which this work reveals checks very well published reports. At the present time the stability of intrinsic factor, in the presence and absence of Vit. B_{12}, is the subject of an extended study and will be reported separately. The combined data on protein and B_{12} binder distribution on our most active preparation permits an estimate of the ultimate level of activity of pure intrinsic factor. In a sample active at a level of 0.5 mg the protein distribution shows three major components, the percent believed to be associated with activity amounting to only 20%. On this basis it can be predicted that the ultimate level of activity for pure intrinsic factor will be of the order of 0.1 mg. It is of interest to estimate how much Vit. B_{12} is bound by pure intrinsic factor, assuming that the particular binder measured at 9.0 cm is the active principle. For this purpose various amounts of tagged Vit. B_{12} were applied to some of our paper strips and autoradiographed. This gave the standard curve shown in Fig. 4. From Table I and Fig. 4 (extrapolated) the area under the 9 cm peak of standard sample 1 corresponds to 1.5 μg of B_{12}. Assuming pure intrinsic factor is active at a level of 0.1 mg, this sample, active at 1.5, mg contains 6.7% intrinsic factor. Since 1 mg of sample was applied to the paper strip, 0.067 mg of intrinsic factor was responsible for binding the 1.5 μg of B_{12}. On this basis one milligram of intrinsic factor will bind 23 μg of Vit. B_{12}. If the molar binding ratio of intrinsic factor to B_{12} is 1:1, molecular weight of intrinsic factor should be around 60,000. These are rough estimates, but these figures for B_{12} binding capacity and molecular weight of intrinsic factor agree well with other published estimates (6,12). The estimates refer to the intact hog intrinsic factor found in mucosal lining of the hog. It is recognized that the active principle may be a much smaller molecule, which in nature is either a component part of a large protein, or bound thereto.

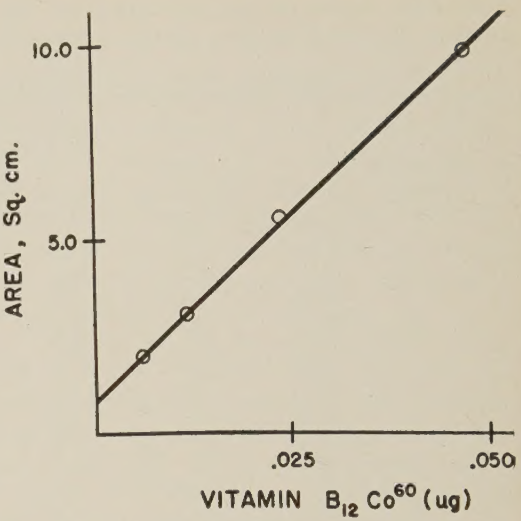

FIG. 4. Autoradiograph response to varying amounts of tagged B_{12}.

Summary. Simultaneous use of paper electrophoresis and radioactive Vit. B_{12} permits separation and quantitation of the various B_{12}

binding components present in commercial intrinsic factor concentrates of hog origin. The amount of one particular binder has been found to correlate with the clinical activity of the concentrates as measured by the urinary excretion method of Schilling. This provides the basis for a simple *in vitro* assay of potency of hog intrinsic factor concentrates ranging in level of activity from 2.0 mg to 75.0 mg. It is predicted that pure hog intrinsic factor will be active clinically at a level of 0.1 mg; calculations suggest that it will bind 23 μg B_{12}/mg and have a molecular weight of 60,000.

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Histochemical Localization and Metabolic Significance of Glucose-6-Phosphate Dehydrogenase System in Adrenal Cortex.* (24959)

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It has been suggested that the adrenal cortex is one of the few mammalian tissues which utilizes the oxidative mechanism of pentose phosphate pathway for metabolism of carbohydrate in preference to the more usual glycolytic pathways of Embden and Myerhof and the citric acid cycle(1,2). Haynes(3,4) provided evidence that adrenocorticotrophin (ACTH) may act to promote steroid synthesis through a stimulating effect on adrenal phosphorylase with increased production of both glucose-1-phosphate and glucose-6-phosphate. The latter compound enters the pentose phosphate pathway *via* a triphosphopyridine nucleotide (TPN) mediated dehydrogenation to 6-phosphogluconic acid, with reduction of TPN to TPNH. The 6-phosphogluconic acid is in turn oxidatively decarboxylated, again using TPN as an electron acceptor with production of ribulose-5-phosphate and reduced TPN (TPNH). The system at this stage is acting as a TPNH generator. This is particularly significant since TPNH is required in the adrenal cortex as reductant for hydroxylations of the steroid molecule. These data in metabolic studies were obtained by quantitative methods which do not preserve structural relationships in the tissue. It was felt that use of histochemical technics for demonstration of some enzyme systems involved might contribute useful data on localization of these systems within the cortex and thus supplement the quantitative observations.

Materials and methods. Adrenal tissue was obtained from 10 250 g, female, white rats fed a standard laboratory diet and acclimated to animal room for at least a week. Animals were sacrificed by blow on head. Both adrenals were removed immediately and 15 μ frozen sections were cut in a cryostat. Each section constituted a cross section of the entire gland. Multiple sections from each gland were taken. Some of these sections were incubated 20 minutes at 37°C in a substrate solution (I) designed to demonstrate activity of the glucose-6-phosphate dehydrogenase system. This solution is composed of 30 mg of potassium salt of glucose-6-phosphate; 5 mg of TPN and 5 mg of a paranitrophenyl substituted ditetrazolium salt (NBT)(5) in 10 ml of 0.1 M veronal buffer at pH 7.4. Potassium cyanide to 0.1 M is added as inhibitor of tissue cytochrome system. The reaction depends on oxidation of glucose-6-phosphate with the passage of electrons to TPN *via* the specific dehydrogenase. TPNH is then reoxidized by means of TPNH diaphorase with the electrons being transferred to the NBT. Reduction of NBT results in formation of a relatively insoluble (fat and water), substantive, blue, granular pigment at site of enzyme activity. It is important to note that a positive reaction is dependent on presence in the tissue of both specific dehydrogenase and TPNH diaphorase activity. Further significant observations can be made on this system by incubating parallel tissue sections for 20 minutes at 37°C in a substrate solution (II) containing 3 mg of TPNH and 5 mg NBT in 10 ml of 0.1 M veronal buffer at pH 7.4. Potassium cyanide to .01 M is added. In this system the reduced coenzyme is used as substrate and thus demonstrates TPNH diaphorase activity directly. If for instance after incubation of a tissue section in substrate I no or little reaction is demonstrated, and if in a parallel section incubated in substrate II a positive reaction for TPNH diaphorase is obtained it can be inferred that the factor lacking for completion of the reaction in substrate I is glucose-6-phosphate dehydrogenase activity. If the reactions in both solutions happen to be negative, one can be certain that diaphorase activity is low or lacking but nothing can be said about the activity of the dehydrogenase.

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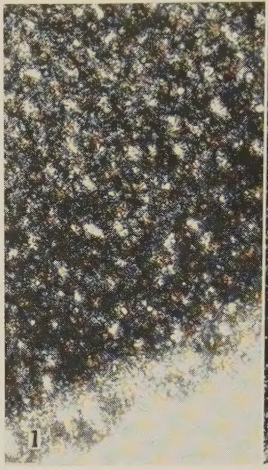

FIG. 1. A section of adrenal cortex incubated in substrate solution I (glucose-6-phosphate; TPN; NBT) showing sharp contrast in activity of glucose-6-phosphate dehydrogenase system between fascicular cortex and glomerular cortex. ×80.

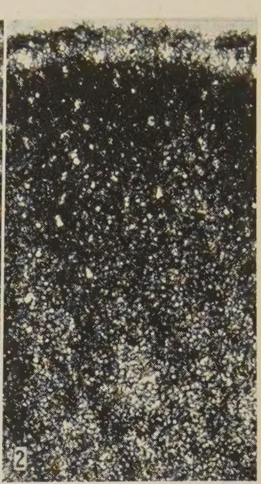

Fig. 2. A section of adrenal cortex taken parallel to Fig. 1 incubated in substrate solution II (TPN; NBT) showing intense activity of TPNH diaphorase system in both fascicular and glomerular cortex. A zone of cells one to 2 cell layers thick lying between fascicular and glomerular zones shows reduced activity. ×53.

Results. Sections of adrenal cortex incubated in substrate solution I (glucose-6-phosphate; TPN; NBT) showed extensive deposition of dye in cytoplasm of cells of the zona fasciculata and reticularis with the greatest concentration present in middle and outer fascicular zone. The glomerulosa, however, showed considerably less activity (Fig. 1). Parallel sections incubated in substrate solution II (TPNH and NBT) also showed intense dye deposition in the fascicular and reticular zones, but in sharp contrast to sections incubated in the first medium, these sections showed marked enzyme activity in the glomerulosa. This indicated presence of considerable TPNH diaphorase activity in the glomerulosa. One could infer that the comparatively poor dye deposition in glomerulosa of those adrenals incubated in substrate solution I was due to a relative deficiency of specific glucose-6-phosphate dehydrogenase activity. These results were obtained in all animals studied.

Discussion. Activity of glucose-6-phosphatase dehydrogenase system was low in the glomerular zone of the adrenal cortex. In contrast, considerable activity of this enzyme system was present in both fascicular and reticular zones. The comparatively low concentrations in the glomerulosa were due at least in part to a relative lack of specific dehydrogenase activity. One can not rule out by this method the possibility that TPN may also be low in the glomerulosa, because TPN is added in reaction I. If as has been suggested one of the roles played by this enzyme system in the cortex is to act as a TPNH generator, providing this reductant for important hydroxylations of the steroid molecule, it is reasonable that it be most active in the fascicular and reticular zones. These are the areas which seem to react most strikingly in situations of acute and chronic stress and following ACTH administration. Morphologic and histochemical criteria of response to stress including ascorbic acid depletion, lipid and cholesterol depletion and hypertrophy all appear to involve the reticular and fascicular zones(6). It has not been demonstrated that the glomerulosa participates actively in these adjustments, at least in the early stages. This zone appears to react demonstrably in a specific fashion to alteration in electrolyte concentration. In the light of our present knowledge it would seem that the fascicular and reticular zones are the areas of the cortex where increased synthesis of steroids occurs in response to acute and chronic stress, and it is here that one would suspect a requirement for increased TPNH and potential TPNH generator systems.

Summary. A histochemical technic was used to localize the activity of glucose-6-phosphate dehydrogenase system in zones of the adrenal cortex. It was very active in the fascicular and reticular zones. Activity in the glomerulosa was comparatively low. Since this enzyme system may be indirectly influenced by ACTH and is a potential provider of TPNH, a reductant important in steroid synthesis, demonstration of its predominance in a zone where active steroid synthesis is presumed to occur is reasonable and of considerable interest.

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Pathway of Enterogastric Reflex.* (24960)

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That acid in upper small intestine inhibits emptying of stomach was first described at end of 19th century by Serdjukov in Pavlov's laboratory(1). This phenomenon present in dogs exists also in human subjects (Barsony and Egan(2)). Thomas and co-workers(3,4, 5) pointed out that delay in gastric evacuation was not due to pylorospasm, but to reduction in motility of the whole stomach. They found the mechanism undisturbed by splanchnicectomy but reduced by division of the vagi. The name "enterogastric reflex" was coined for the phenomenon observed. Quigley *et al.*(6,7) reported similar findings in that vagotomy elevated the threshold for acid inhibition of the stomach. In human subjects we found the inhibitory effect of acid in the duodenum or upper jejunum, as measured by balloon-transducer-recorder system, persisted after complete supradiaphragmatic vagotomy, thoracolumbar sympathectomy, partial gastric resection, or transection of

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* This work supported by U. S. Public Health Grant.

TABLE I. Inhibition of Gastric Motility by Acid in Jejunum.

Vol 0.1 N HCl (cc)	Duration of inhibition (min.)
10	1– 5
20	5–16
30	10–35
40	13–30
50	25–40
60	22–40
70	28–55
80	55–60
90	40
100	30–55

TABLE II. Effect of Vagotomy on Enterogastric reflex.

Vol 0.1 N HCl (cc)	Duration of inhibition (min.)
10	4– 6
20	5–12
30	6–18
40	8–15
50	10–21
60	10–20
70	35–40

spinal cord(8). The inhibitory effect of acid varied as a linear function of hydrogen ions introduced(9). Since vagus nerves did not seem of primary importance in the reflex arc responsible for acid inhibition of the stomach, the following experiment was performed on dogs.

Method. Sixteen dogs were prepared with Maydl fistula of proximal jejunum plus cannula gastrostomy and Heidenhain pouch. Motility was recorded from either or both vagally innervated main stomach and vagally denervated Heidenhain pouch, using a balloon introduced through cannula and connected to Statham pressure transducer and Sanborn twin-viso recorder. Acid in varying amounts was introduced through a catheter threaded into the jejunum through the Maydl fistula. Volumes of 0.1 N HCl varying from 10 to 100 cc were infused into the jejunum and duration of inhibition of gastric motility measured. In 2 dogs complete bilateral thoracolumbar sympathectomy was performed. In 8 dogs bilateral supradiaphragmatic vagotomy was done. In 3 dogs both vagotomy and thoracolumbar sympathectomy were completed. In 3 additional animals the celiac plexus was destroyed by dissecting clean the celiac and superior mesenteric arteries and the adjacent aorta, followed by application of 5% phenol solution to the area. Following these procedures in 16 dogs the same doses of 0.1 N HCl were infused into the jejunum while gastric motility was recorded.

Results. Control studies in 16 animals demonstrated that, as in the human, duration of gastric inhibition increased progressively with amount of HCl infused into the jejunum. Increases above 70 cc however, did not significantly increase period of inhibition. The results are summarized in Table I and an example presented in Fig. 1. In each of 32 studies performed, the same volume of 0.9% NaCl solution infused into the jejunum at the same rate failed to produce inhibition of gastric contractions. Duration of inhibition observed when recordings were made from the Heidenhain pouch was not significantly different from that observed in the main stomach, even where simultaneous recordings were made. The latent period between infusion of HCl and gastric inhibition varied from 10 to 30 seconds.

In 8 vagotomized dogs, 29 studies were performed following complete recovery. Inhibition of gastric motility was observed in all studies. The latent period between infusion and inhibition varied from 1 to 4 minutes, a slight increase over that observed in control studies. Duration of inhibition was, however, only slightly less than that seen with same volumes of infusion in the unvagotomized animals. The studies are summarized in Table II.

Eight studies were performed in 2 dogs following bilateral thoracolumbar sympathectomy. Latent period and duration of inhibition were unchanged over those observed in control studies on the same animals. Acid infusions in dogs with both vagotomy and lumbodorsal sympathectomy were entirely similar to those with vagotomy alone.

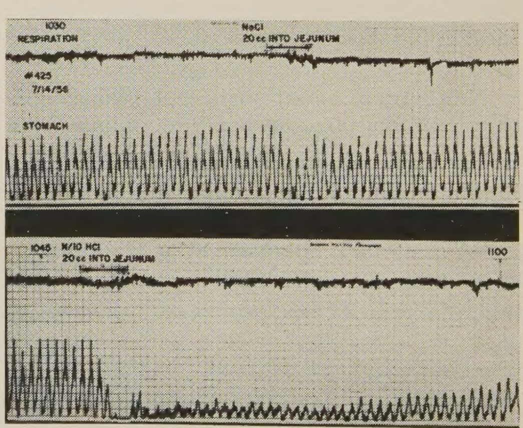

FIG. 1. Inhibition of gastric motility by acid in jejunum.

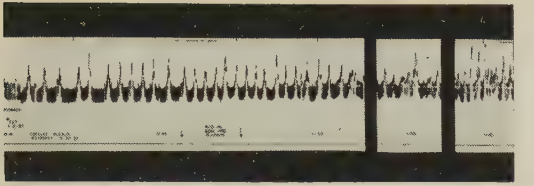

FIG. 2. Failure of acid in jejunum to inhibit gastric motility after destruction of celiac plexus.

Following celiac ganglionectomy, 8 studies were performed in 3 dogs with infusion of 10 to 40 cc of 0.1 N HCl into the jejunum. In no experiment was inhibition of gastric contractions observed. A typical example is presented in Fig. 2. When 10 cc of olive oil was infused into the jejunum, however, profound and prolonged inhibition of gastric motility was observed after latent period of 10 to 20 minutes.

Discussion. Inhibition of gastric motility as recorded from a Heidenhain pouch after infusion of dilute HCl into a jejunal fistula, was entirely comparable to that observed when contractions were recorded from the main stomach. Since the Heidenhain pouch is without vagal innervation, this observation indicates the vagi are not implicated in the enterogastric reflex. Following bilateral supradiaphragmatic vagotomy, the same inhibition from HCl in the small intestine was observed although latent peirod was longer and period of inhibition slightly less. This difference might be explained by general decrease in tonus and motility of stomach following complete vagal section. Complete bilateral thoracolumbar sympathectomy did not influence the enterogastric reflex and even when combined with bilateral vagotomy the phenomenon persisted. These data indicate that pre-ganglionic sympathetics and their spinal cord connections are not involved in the pathway of the enterogastric reflex.

Celiac ganglionectomy completely abolished inhibition of gastric motility resulting from 0.1 N HCl infusions into the proximal jejunum. These observations compare with those of Simba(10), who produced gastric inhibition by application of hypertonic saline to intestinal loops. This effect also was not influenced by vagotomy or sympathectomy, but was blocked by celiac ganglionectomy. This indicates that gastric inhibition resulting from intestinal hyperperistalsis, and perhaps intestinal distention, probably has the same nervous pathway through the post-ganglionic sympathetics as does acid inhibition.

Summary. Acid in upper small intestine causes immediate inhibition of gastric motility. Sixteen dogs were prepared with gastric fistula and/or Heidenhain pouch plus a Maydl fistula of upper jejunum. After control studies, the animals were (1) vagotomized; (2) totally sympathectomized; (3) both vagotomized and sympathectomized or (4) celiac ganglionectomized. Inhibitory effect of acid in the jejunum on gastric motility was still present after either or both vagotomy and sympathectomy. However, no inhibition was produced after celiac ganglionectomy. This study indicates the pathway for enterogastric reflex is through the post ganglionic sympathetics.

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Preparation of High Potency Poliovirus in FL Cell Cultures at Room Temperature.* (24961)

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It was shown in earlier studies(1) of the reproduction of poliovirus at 37°C in various cell strains of human derivation that appreciable quantities of infectious virus appear in cells about 6 hours after infection; the titer increases rapidly thereafter and reaches a peak at about 12 hours. The intracellular concentration of virus then declines quickly due to release into the culture fluid. Highly potent preparations of virus can be harvested from the infected cells by mechanical disruption in a Mickle tissue disintegrator, but the harvest must be timed carefully if a good yield is desired. In the present study it has been found that during cultivation at 32°C, virus accumulates almost as rapidly and reaches practically the same intracellular titer as at 37°C. However, release of virus at 32°C is much slower than at 37°C and as a result, the intracellular concentration of virus remains at or near the peak level for 4 to 6 hours. For purposes of production of highly potent virus by mechanical disruption of infected cells it is more convenient to operate at lower temperature, since the need for careful timing of harvest is thus obviated. Furthermore, it has been found that careful control of temperature is unnecessary, since potent virus preparations for use in immunological studies have been obtained consistently by cultivation at room temperature for approximately 18 to 20 hours, followed by extraction of virus from infected cells.

Materials and methods. Poliovirus Type 1, Mahoney strain, was used. Virus infectivity assays were made by plaque technic in rhesus monkey kidney cultures, viral antigen was assayed by complement fixation (CF) tests with human convalescent sera, and FL cells for virus propagation were grown as described elsewhere(1,2).

Results. Kinetics of virus formation and release at 37° and 32°C. Four days after seeding, 25 replicate 32 oz bottle cultures of FL cells were washed 3 times with 10 ml portions of maintenance fluid and infected by addition of 25 ml of maintenance fluid containing sufficient virus to provide a multiplicity of 28 plaque forming units (PFU)/cell. After 3 hours incubation at 37°C the maintenance fluid was removed and cultures washed 3 times with 10 ml portions of maintenance fluid. Ten cultures were incubated at 32°C and another 10 bottles were kept at 37°C. The remaining 5 cultures were used for counts of cell nuclei(3). At intervals shown in Fig. 1, samples consisting of one culture were removed from respective incubators. The fluid phase was withdrawn and assayed for infectivity. The cells were scraped off with a rubber tipped glass rod, suspended in chilled veronal buffer(1) and centrifuged at 2000 rpm for 2 minutes. The supernate was discarded. The cells were resuspended in 1 ml of veronal buffer, disrupted in Mickle Tissue Disintegrator as described previously(1), and extracts so obtained were assayed for virus infectivity. The results, expressed in terms of PFU/cell, are shown in Fig. 1.

It is evident that decrease in temperature affects release of virus much more markedly than its formation. As a result, a high intracellular concentration of virus persists for 4

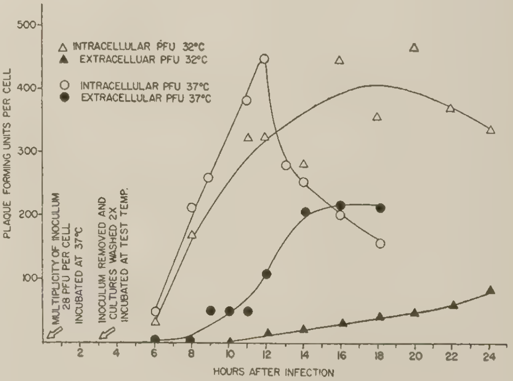

FIG. 1. Formation and release of poliovirus type 1 in FL cells incubated at 37 and 32°C.

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* Aided by grant from National Fn.

TABLE I. Assay of Intracellular Virus Prepared from Infected FL Cells Maintained at Room Temperature Overnight (18-20 Hr), 4 Lots.

Lot #	No. of 32 oz cultures	Vol of cell extract, ml	PFU/ml	PFU total
1	10	3	1.08×10^{10}	3.2×10^{10}
4	18	5	.92 ”	4.6 ”
5	10	3	1.12 ”	3.36 ”
8*	7	2.43	.91 ”	2.21 ”

* Material was ultracentrifuged in sucrose density gradient prior to assay (Zone SDG^D).

to 6 hours at 32°C and good harvest can be obtained at any time during that interval.

Preparation of high potency virus material from infected cells incubated at room temperature. Five to twenty 32 oz bottle cultures of FL cells were inoculated at one time, each with 25 ml of maintenance fluid containing sufficient virus to provide a multiplicity of 20-30 PFU/cell. Cultures were incubated at 37°C for 2 to 3 hours. The maintenance fluid containing the inoculum was removed and 25 ml of fresh maintenance fluid was added. The cultures were then placed on top of a laboratory bench for 18-20 hours. A recording thermometer in the same location indicated a temperature range 26° to 32°C. At time of harvest the tissue culture fluid was discarded; the cells were scraped off, pooled, and packed by centrifugation at 1000 rpm for 4 minutes. The packed cells were stored in a glass vial at −55°C until needed.

Several lots of infected cells were thus prepared. All lots were made with an inoculum of virus prepared from cells infected and maintained at 37°C. On several occasions the virus inoculum was reused after replenishing for virus lost by adsorption to cells or by inactivation. Assays made on extracts from 4 lots of infected cells are shown in Table I. Lot #8 was fractionated by ultracentrifugation in a sucrose density gradient(2,4). CF tests of the SDG^D fraction of the sucrose density gradient with a selected monoreactive convalescent serum of *D* specificity indicated the presence of approximately 1000 CF units of *D* antigen(4). From the numerical relationship established previously(1,2) between CF activity of the antigen in the SDG^D zone and physical virus particles(2,3), it was computed that SDG^D contained 10^{13} physical virus particles, of which at least 2.21 x 10^{10}, *i.e.,* about 1 in 400, were infectious for rhesus monkey kidney monolayer cultures. Because of low sensitivity of this assay system as compared with other assays(5,6), as well as for other reasons stated elsewhere(1), the infectious virus content is probably much higher.

Summary. Rate of formation and release of poliovirus Type 1 were studied at 32° and 37°C in FL cell cultures. Virus accumulated almost as rapidly and reached practically the same intracellular levels at 32°C as at 37°C. However, release of virus was much slower at 32° than at 37°C and as a result, at 32°C the intracellular concentration of virus remained at or near peak levels for 4 to 6 hours. From these observations, highly potent virus for use in immunological studies was prepared by mechanical disruption of infected cells maintained at room temperature (26-32°C) for 18-20 hours.

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Continuous Grouth of *Brucella abortus* in Mononuclear Phagocytes of Rats and Guinea Pigs.* (24962)

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In naturally acquired bacterial infections, infecting organisms tend to be passed from host to host without growth on lifeless media. This may at least in part contribute to the maintenance and production of proper antigenic and physiologic states required for establishment of the parasite in the new host. In studying factors of major importance in pathogenesis and control of naturally acquired brucellosis, it would therefore appear likely that, in comparison with organisms grown on lifeless media, *Brucella abortus* organisms grown intracellularly might better approximate the state of organisms as ordinarily transmitted. Recent studies(1-4) have demonstrated that such intracellular growth can be accomplished under *in vitro* conditions by permitting brucellae to multiply with mononuclear phagocytes maintained in tissue culture. However, all prior studies have been concerned with only one cycle of cultivation under these conditions. This may not have sufficed to produce those physiologic and genetic states of importance in natural disease. Therefore, this study was undertaken to determine if continuous passage of *Br. abortus* in mammalian mononuclear cells is possible. Results indicate that such continuous passage without intervening cultivation on lifeless media can indeed be accomplished. In addition, it has been observed that mammalian species supplying host cells significantly affects the outcome of such continuous intracellular cultivation.

Methods. Br. abortus, strain SA-S, isolated long ago from a human case in Puerto Rico, and its rough variant SA-R(3) were studied. Guinea pig and rat mononuclear cell cultures were prepared according to method of Pomales-Lebrón and Stinebring(1). Normal, young, male guinea pigs of the Hartley strain and normal adult female white rats provided the mononuclear cells. For harvesting these cells, the peritoneal cavity of animal was washed with Hanks' solution 2 days after intraperitoneal injection of 20 ml of saline. Approximately 1 x 10^7 of washed mononuclear cells were mixed with approximately 2.5 x 10^8 brucellae in 5 ml of 30% autologous serum-Hanks' medium. The mixture was pipetted into a T-30 flask and incubated 2 hours. During this time, mononuclear cells containing ingested brucellae attached themselves to the floor of flask. The supernate was then replaced with identical medium supplemented with 10 μg of streptomycin/ml. This concentration of antibiotic has been proved capable of killing extracellular brucellae but not intracellular organisms. Three days later cultures were gently washed twice with warm Hanks' solution to remove residual streptomycin, small glass beads[†] and 8 ml of Hanks' solution were added, and the flasks shaken with rotating motion of the hand. This procedure disrupted the cells without affecting the brucellae. A portion (3.5 ml) of resulting supernate containing the bacterial harvest was immediately added to 1.5 ml of a fresh monocyte-serum mixture, thus initiating the next cell culture. Viable counts on aliquots of harvest were done on tryptose agar.

Results. Continuous transfer of Br. abortus in guinea pig cells. The data in Fig. 1 and Table I demonstrate that it was possible to transfer smooth organisms of *Br. abortus* through monocytes for more than 20 transfers without intervening growth on lifeless media. As far as yield of viable bacteria/monocyte culture was concerned, there was an initial period of continually increasing viable count, followed by period of relative stability. Approximately 10^8 organisms/culture appeared the maximum yield. Poor harvests were obtained whenever microscopic observation in-

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* These studies were supported by Contract with the U. S. Army Chemical Corps.

[†] Minnesota Mining and Manufacturing Co. 3M Superbrite No. 100.

TABLE I. Harvest (per Flask) of *Brucella abortus* Passaged in Continuous Transfer.

Passage	SA-S, guinea pig cells	SA-S, rat cells	SA-R, guinea pig cells
0	1.7×10^6	2.0×10^6	5.2×10^7
1	2.5 "	5.2 "	6.0×10^6
2	6.0 "	1.8×10^7	4.0×10^3
3	2.2×10^7	9.6×10^6	$<1.0 \times 10^2$
4	6.8 "	2.4 "	Discontinued
5	1.9×10^8	1.0 "	
6	2.7 "	3.6×10^5	
7	1.6 "	1.1×10^6	
8	1.2 "	2.5×10^5	
9	1.8 "	1.2 "	
10	6.8×10^7	4.8×10^3	
11	2.2 "	2.3 "	
12	5.2 "	2.0 "	
13	6.4 "	1.0 "	
14	6.4 "	$<1.0 \times 10^2$	
15	5.6×10^6	Discontinued	
20	3.2 "		
25	8.1×10^7		
26	1.8×10^8		
27	8.4×10^7		

dicated a poor state of tissue cultures. Similar findings were obtained with 3 other definitive but less extensive transfer series. In the experiment here described, contamination occurred at 27th transfer, forcing discontinuation of the series. The results justify the assumption that intracellular growth involving transfer through guinea pig monocytes can be maintained practically indefinitely.

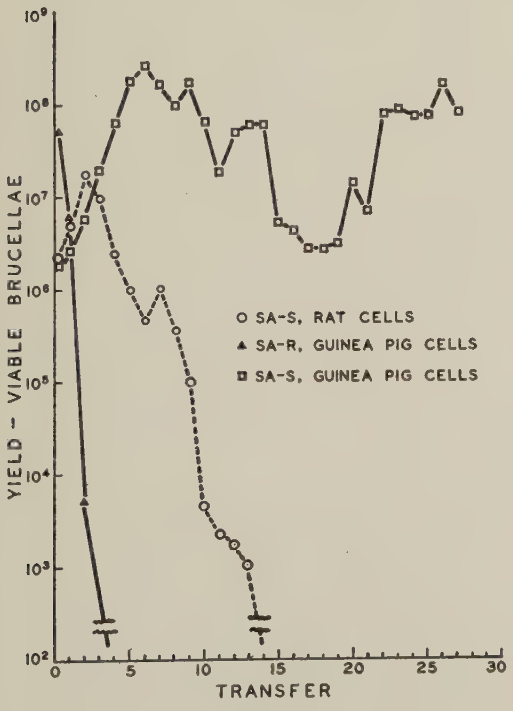

FIG. 1.

Differences between growth of Br. abortus *in guinea pig- and rat-cell cultures.* Fig. 1 and Table I show that in our system, at least, rat mononuclear cell cultures do not support continued growth of SA-S in a manner comparable to that observed during continuous transfer in guinea pig cells. A gradual decline of viable count with complete loss of recoverable brucellae by transfer 14 occurred. Similar trends were observed in 2 other experimental series. It should be mentioned that rat cells kept under our culture conditions generally appeared more rounded than healthy guinea pig monocytes. This is usually indicative of cells in poor condition. Based upon our experience with guinea pig monocytes, however, these changes were not of sufficient magnitude to account for the striking decrease in brucellae observed in rat-cell transfers.

Lack of growth of rough variants. Prior investigations(3) had demonstrated inability of avirulent rough *Br. abortus* organisms to multiply in guinea pig mononuclear cells. This was again confirmed by observations that SA-R bacteria are rapidly eliminated from guinea pig- and rat-cell cultures (Fig. 1) and Table I. This evidence reemphasizes the utility of the present method in demonstrating strain differences in ability to survive and multiply in intracellular environment.

Cultural characteristics of isolates. To check for possible occurrence of changes in characteristics following continuous multiplication in an intracellular environment, tests were made on stability of a number of easily detectable characteristics of bacteria harvested after various periods of continuous transfer through monocytes. The characteristics tested included (1) possible changes in colonial morphology, as detected by plating of aliquots of the harvest fluid directly onto tryptose agar with and without triphenyltetrazolium or onto 2-1 agar, (2) sensitivity to thionin and basic fuchsin, (3) capability of growing in air and CO_2 (SA-S is an aerobic mutant), and (4) agglutinability by specific antiserum. No detectable alterations in these characteristics were found. However, the potential for population changes under conditions employed was indicated by the fact that a few rough (R) colonies present in the initial predominantly smooth (S) inocula disappeared after one or 2 transfers, suggesting that the technic of continuous transfer is capable of selecting out S types from a mixed population. Other changes, such as alterations in virulence and immunogenic characteristics may have occurred during these transfers, but their detection requires more extensive tests, now under way.

Discussion. Brucellae are capable of surviving and multiplying within the cytoplasm of certain host cells(5,6). Therefore, continuous growth in a corresponding micro-environment *in vitro* may provide means for maintaining the organism in a physiologic and genetic state approximating that found in naturally infected animals. As demonstrated with some bacteria(7), antigenic differences between genetically apparently identical organisms grown under different environmental conditions can occur and, therefore, differences between slant-grown and intracellularly grown brucella could be expected. Although detection of such possible differences will require further work, the present study has led to development of a method for investigating these and related factors of importance in pathogenesis.

Results of comparison of growth within guinea pig mononuclear leukocytes and rat mononuclear leukocytes correlate with what is known about the *in vivo* fate of *Br. abortus* in these animals. Guinea pigs are susceptible to small numbers of S. brucellae, and the organisms can be recovered from spleen or lymph nodes long after initiation of infection. In contrast, rats seem to possess a higher degree of natural resistance(8,9). Correlation between such information and our results suggests that resistance at the cellular level may be of considerable importance in determining such differences in host susceptibility. The slow loss of brucellae in the rat mononuclear system may indicate that subtle interference with intracellular multiplication, rather than strong bactericidal factors, is involved. Thus, in the rat, innate resistance to brucellosis may depend, at least in part, upon innate activity of the phagocytic cells themselves. Later in the disease, immune factors may become more important. On the other hand, in the guinea pig, whose exposure to small numbers of virulent bacteria results in a progressive but, eventually, limited disease, resistance may depend upon *subsequent* modification of state of resistance of the phagocytic cell. It has been shown that virulent brucellae are capable of multiplying within monocytes from normal guinea pigs, but multiplication is inhibited within monocytes from infected or immune animals(1). Thus, in a susceptible host such as the guinea pig, the brucellae initially depend on favorable intracellular environment for nutrients, but subsequently encounter an interference with multiplication. This interpretation of present data presumes that no significant modifications of the mononuclear cells have occurred during their *in vitro* maintenance which would alter resistance factors so that extrapolation to *in vivo* conditions would be misleading.

The basis for the observed interference with bacterial multiplication in rat mononuclear cells remains a matter for speculation. It may involve effective yet weak antibacterial intracellular factors, it may also be due to a lack of an essential nutrient for bacterial multiplication, or it may involve some other metabolic factor. In connection with the latter possibility, it is interesting to note that Wallace and his associates(10) have demonstrated a profound effect of hydrocortisone on intracellular multiplication of *Mycobacterium lepraemurium*, an effect that is presumably due to modification of metabolism of the infected cell and that correlates well with the known effects of this and related hormones in certain diseases.

In apparent contrast to our observations, Holland and Pickett(4) have reported growth of brucellae in rat mononuclear cells. However, we have noted similar evidence of multiplication in non-continuous rat-cell cultures. This may be an expression of a limited amount of residual intracellular growth following growth on lifeless media. It should be remembered that only a few cell divisions are required to pack a mononuclear cell with bacteria.

Summary. Continuous intracellular growth of virulent *Brucella abortus* organisms, within mononuclear cells of guinea pigs, by periodic transfer from tissue culture to tissue culture has been accomplished. Comparable continuous growth could not be accomplished using rat-monocyte cultures. Continuous transfers were unsuccessful in the case of rough avirulent organisms in guinea pig- and rat-monocyte cultures. The bearing of these observations upon host and species resistance has been discussed.

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Virologic Use of Monkey Kidney Cells Preserved by Freezing.*† (24963)

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Because of their susceptibility to a broad spectrum of viruses affecting man, as well as their necessity in production of vaccines for human use, cultured Rhesus monkey kidney cells continue to serve as an essential virologic tool. There are, however, many well-known difficulties involved in their use, such as availability and viral infections acquired *in vivo*(1), which might be circumvented if such cells could be preserved until they are needed or tested. Ability to preserve mammalian cell strains(2-5) and certain animal tumors(5) by freezing suggested that intact cells enzymatically separated from their host tissues might be successfully treated in a similar fashion. This communication describes a procedure of freezing and storage as efficient and practical means of preserving cells of freshly trypsinized Rhesus monkey kidneys as well as cells of primary cultures. Also described are their sensitivities to a variety of viruses as determined by cytologic response and assays by tube titrations or plaque counts.

Procedures. Preparation of cell suspensions. Two types of cell suspensions were frozen: 1) freshly trypsinized Rhesus monkey kidney prepared in the laboratories of Parke, Davis & Co., by methods routinely employed for production of virus vaccines, and 2) versene-treated monolayers of primary cultures of monkey kidney that had been grown in Eagle's basal medium containing 5% calf serum. In both cases the cells were resuspended in freeze medium which consisted of Eagle's basal medium 80%, calf serum 15%, and glycerol 5%. Concentrations of living cells were determined by adding 0.1 ml of 0.1% solution of neutral red to 1 ml of cell suspension, and the mixture incubated 30 minutes at 37°C. Up to 4 ml aliquots containing 0.8 to 2 x 10^6 living cells/ml were transferred to 5 ml vials and sealed with apron rubber stoppers. Sufficient concentrations of cells were frozen to

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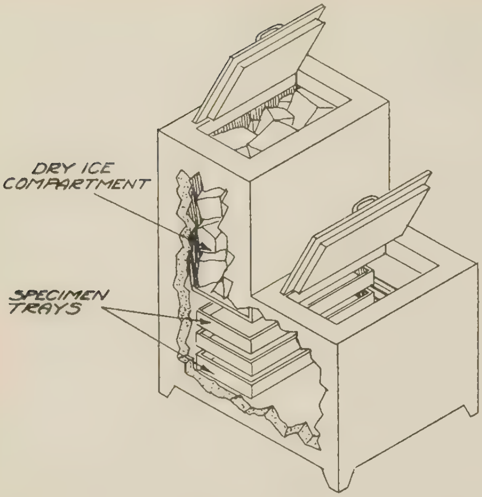

FIG. 1. Dry ice chest to maintain temperature of –75°C ± 2°C. Specimen trays slide forward to lower opening for rapid location of individual vials.

allow at least 1:10 dilution of glycerol after thawing, thereby eliminating the necessity for removing the glycerol before culturing. *Freeze-thaw procedure.* Following the procedure described by Stulberg, *et al.* for cell strains(2), the vials were frozen in controlled-rate slow freeze apparatus‡ with temperature drop of 1°C/minute. They were removed at –25°C and transferred to dry ice cabinet (Fig. 1) designed to maintain a permanent storage temperature of –75°C ± 2°C. It has been shown that at temperatures above –50°C, stored cells may become non-viable(2-4). Frozen cells were rapidly thawed under running water at 37°C and the thawed cells were again enumerated after staining with neutral red. *Culture procedures for thawed cells.* Suspensions of thawed cells were appropriately diluted with growth medium which consisted of Eagle's basal medium containing 5% calf serum and antibiotics. Tubes were implanted with 1.5 x 10^5 cells in 1 ml of medium, and 50 mm Petri dishes with 1.5 x 10^6 cells in 7 ml of medium. If the cells were to be incubated in a 5% CO_2 95% air atmosphere, the medium contained 2.2 g/l $NaHCO_3$ to maintain pH of approximately 7.2. This was necessary with open vessels such as Petri plates but also was useful with tubes, because under these conditions the thawed cells of freshly trypsinized kidney seemed to attach to glass more rapidly than without 5% CO_2. Tubes were stoppered and removed to ordinary incubation after islands of cells appeared. However no difficulty was encountered with initially stoppered tubes and bottle cultures if a low bicarbonate content of 0.35 g/l was employed. With cultures of thawed cells of freshly trypsinized kidney, the medium was renewed on 6th or 7th day, whereas with thawed cells of versenated primary cultures, the medium was usually renewed on 2nd or 3rd day of culture. *Virologic procedures.* General susceptibility to a large variety of viruses was determined by cytopathology resulting after inoculation of 100 TCD_{50} of virus into tube cultures. Medium used in tests with various enteroviruses was Eagle's basal medium plus 5% calf serum. With all other viruses, the maintenance medium was Medium 199 plus 2% calf serum. 50% endpoints by tube titration (5 tubes/dilution) were compared in cultures from frozen and non-frozen cells. Plaque forming units were determined by modification of Dulbecco and Vogt(6) technic using overlay (5 ml/plate), consisting of 1 part Eagle's medium plus 5% calf serum (2x concentrated) and 1 part Noble agar (2%). After 72 hours incubation in 5% CO_2, 0.1 ml of 0.5% neutral red solution was added and plaques counted after additional 2-3 hour incubation in 5% CO_2.

Results. Recovery of thawed cells. Numbers of viable monkey kidney cells as determined by neutral red staining that could be recovered immediately after thawing are illustrated in Table I. About 70% recovery

TABLE I. Comparative Counts of Monkey Kidney Cells before and after Freezing. (Neutral red stained.)

Type of cell suspension	Cell counts × 10^5 Frozen	Thawed	Recovery, %
Freshly trypsinized kidney	7.8	5.4	70
Idem	9.3	6.4	69
Versenated primary culture	11.0	11.0	100
Idem	12.0	8.9	75

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‡ Manufactured by Canal Industrial Corp., Bethesda, Md.

was obtained after freezing freshly trypsinized kidney cells and 75-100% recovery of frozen versenated cells of primary cultures. These figures were consistent in over 25 separate batches of cells frozen.

Growth of thawed cells. Tube cultures and Petri dish cultures prepared from frozen freshly trypsinized kidney cells formed confluent monolayers in 8-10 days. These took approximately one day longer to develop than comparable unfrozen cell cultures. Similar to their unfrozen counterparts, tube cultures and petri dish cultures made from frozen-thawed cells of primary cultures proliferated much more rapidly than thawed unpassaged cells, forming confluent monolayers in 2-4 days. In Fig. 2 are photomicrographs of both types of both types of cultures at various periods following their initiation with thawed cells.

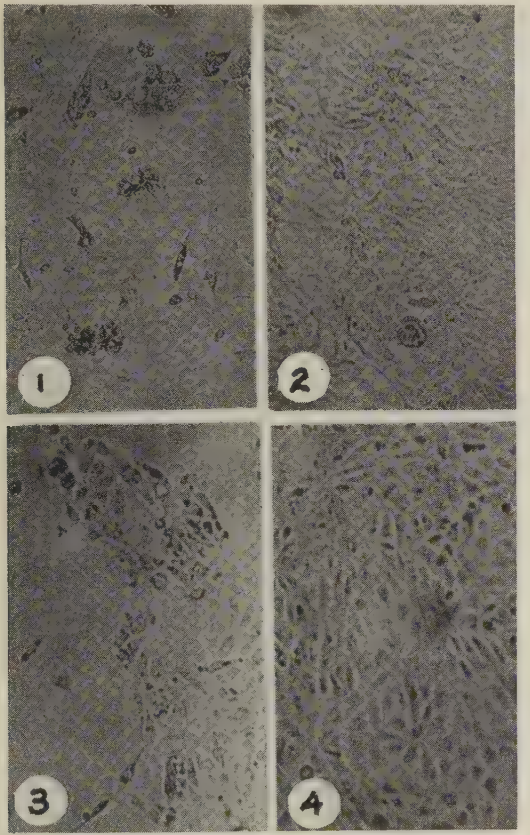

FIG. 2. Appearance of cultures of thawed monkey kidney cells. Trypsinized kidney: (1) after 4 days, (2) 8 days. Primary cultures: (3) after 1 day, (4) 4 days.

TABLE II. Viruses Producing Characteristic Cytopathology in Cultures Made from Frozen Monkey Kidney Cells.

	Cells frozen	
Virus	Trypsinized kidney	Primary culture
Polio—1, 2, 3	+	+
Coxsackie B, 1-5	+	
ECHO, 1-19		+
Adenovirus—7	+	
Influenza A (G.L. 1134)	+	+
Asian	+	
B (G.L. 2239)	+	
CA	+	
HA—1	+	
JH; 2060	+	+
Herpes simplex	+	+
Measles	+	

Susceptibility of preserved cells to viruses. Table II shows sensitivities of preserved monkey kidney cells to 37 different viruses affecting man. Each virus induced cytopathology in cultures of preserved cells which was undistinguishable from characteristic cytopathology produced in cultures of unfrozen monkey kidney cells. Where tested, no differences were observed between cultures of frozen trypsinized kidney and frozen primary cultures. Use of preserved cells for isolation of various enteroviruses from clinical specimens was demonstrated by parallel isolations in cultures of frozen and unfrozen cells of poliovirus-1, Coxsackie A-9 and B-5, and ECHO 9, from stool specimens, and Coxsackie A-9 and B-5 from cerebrospinal fluids.

Virus assay in frozen and unfrozen kidney cells. Table III illustrates a variety of viruses, propagated in monkey kidney cultures and then assayed by tube titration in cultures made from both frozen and unfrozen cells. In

TABLE III. Comparative Susceptibility of Frozen and Unfrozen Cells to Viruses.

	Titer*	
Virus	Frozen cells	Unfrozen cells
Influenza A (G.L. 1134)	1.5	1.5
Asian	4.7	4.7
B (G.L. 2239)	4.0	3.3
HA—1	3.0	2.8
CA	5.5	5.0
JH	5.0	3.5
2060	4.0	3.0
Adenovirus—7	6.5	4.5
Polio—1	8.0	8.0
Measles	3.7	4.0

* Log TCD_{50}/0.5 ml.

TABLE IV. Comparative Plaque Titers on Frozen and Unfrozen Cells.

Virus	Dilution	Frozen No. plaques*	Frozen PFU/ml†	Unfrozen No. plaques*	Unfrozen PFU/ml†
Polio—1	10^{-6}	17, 17, 21, 23, 23	8.3	12, 17, 20, 20, 23	8.0
	10^{-7}	1, 2, 3, 3, 4	8.1	1, 1, 2, 3, 5	8.1
ECHO—7	10^{-5}	39, 41	7.3	40, 42	7.3

* 0.2 ml/plate. † Log.

each type of culture 50% endpoints were comparable and for certain viruses significantly greater in preserved cells. Table IV shows several enteroviruses assayed by plaque technic in plate cultures made from frozen and unfrozen cells. It is evident that plaque titers were comparable in both types of cultures. In addition, ECHO virus serotypes 1, 4, 7, 8, 12, and 13 and Coxsackie B-1 and B-3 were tested for plaque formation. With these, plaques in cultures prepared from frozen cells were indistinguishable from those in unfrozen cells.

Discussion. These results demonstrate that slow freeze procedures and storage at low temperatures, effective for preservation of cell strains, can also be utilized for preserving cells of trypsinized monkey kidney. Rate of freezing, storage temperature, and incorporation of glycerol as an intracellular water binder, are considered essential factors in the procedure. There is general agreement as to optimal rate of freezing (approximately 1°C/minute) and necessity for storage below –70°C. There is some disagreement, however, concerning optimal concentration of glycerol for cell protection through the critical freezing range described by Mitchell, *et al.*(5). Although various cell strains may sometimes tolerate(2-5), and transplantable tumors may even necessitate(5), glycerol concentrations greater than 5%, the trypsinized tissue cells described herein and cell strains reported previously(2), were adequately protected by 5% glycerol. Use of latter concentration has the added advantage of eliminating separate removal of glycerol after thawing, provided sufficient dilution can be employed before culturing.

Ability to preserve monkey kidney cells circumvents a vexing problem encountered in their culture, *e.g.*, simian virus infections acquired *in vivo*(1). Such agents were encountered in a large proportion of batches frozen and were detected in cultures of thawed cells by prolongation of monolayer formation or by characteristic cellular degeneration transmissible to other virus-free cultures.

The practical advantages of preparation directly from thawed cells of cultures for virologic use are self-evident. Freezing had no discernible effect on susceptibility of monkey kidney cells to the large variety of viruses tested. The data show that cytopathology, and assay endpoints by tube titration or plaque counts, in cultures made from frozen cells were indistinguishable from those made from unfrozen cells.

Summary. Cells of freshly trypsinized monkey kidney and of primary cultures of monkey kidney were preserved by slow freezing in 5% glycerol medium and storage at –75°C. Cultures of thawed cells yielded monolayers that could be directly utilized for virologic work. In these, the cytologic response of plaque morphology due to polioviruses, ECHO, Coxsackie, measles, herpes simplex, influenza, CA, HA-1, JH, 2060, and adenovirus, as well as assays by tube titration or plaque counts, were equivalent to those produced in unfrozen cells. Moreover, isolations of ECHO 9, Coxsackie A-9, B-5, and polioviruses, from stools or cerebrospinal fluids were made with equal facility in either type of culture.

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Influence of N-Methylformamide on C^{14}-Formate Incorporation III. Effect of Time of Administration of Isotope. (25964)

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Our previous work(1,2,3) has shown that N-methyl-formamide (NMF) has marked effects on formate utilization in rat livers and tumors. These effects, under particular conditions of the experiments, consist of a large stimulation of incorporation of formate into the nucleic acids, glycogen and protein of the liver, and inhibition of incorporation of formate into nucleic acids of tumor tissue. The conditions under which these effects were observed were: the isotope was given 24 hours after a single large dose of NMF, and the animals sacrificed 24 hours after administration of isotope. When this work was first presented (2) the question was raised as to whether the same type of result would have been obtained at short time intervals after injection of isotope during period of most rapid incorporation. Our data show that the NMF effect is as striking 2 and 6 hours after administration of isotope as at 24 hours. In addition, it was observed, and confirmed, that incorporation of radioactive formate into rat liver nucleic acids follows a multi-peak curve, with a minimum around 4 to 6 hours after injection of isotope.

Methods. Male Wistar strain rats (Carworth Farms) weighing 150 ± 10 g were used. Nine rats were injected, at approximately the same time, with a single dose of 2 g/kg of body weight of NMF* administered as 50% solution in physiological saline). Each rat was injected, in groups of 3, with 4 μc of C^{14}-formate,† one group 24 hours after NMF, another at 42 hours and the other 46 hours after NMF injection, appropriate controls of 3 rats/group being injected with isotope at corresponding times. They were all sacrificed 48 hours after injection of NMF and livers of each group pooled in dry ice. Livers were first homogenized in alcohol, alcohol-ether, and ether in Waring blendor and these extracts discarded. The solids were then extracted in the cold 3 times with 5% trichloroacetic acid (TCA) and glycogen isolated from pooled TCA extracts by alcohol precipitation. Glycogen samples were recrystallized to constant radioactivity, and assayed for purity by the anthrone reaction(4). They all varied between 98-101%, using glucose as standard. Nucleic acids were next extracted and individual purines isolated as described previously (1,3). The residues from this extraction were stirred well with 5% TCA at 85°, filtered and washed well with TCA, water, alcohol and ether. These final residues were ground to a fine powder, suspended in 25% ethanol and plated on planchets for determining protein radioactivity. Activities are presented as relative specific activities (RSA) where

$$\text{RSA} = \frac{\text{counts/min./}\mu\text{M isolated compound}}{\text{counts/min./}\mu\text{M injected compound}} \times 100$$

in the case of purines. In the case of combined nucleic acids, glycogen and protein, RSA was calculated on a mg basis.

Results. Fig. 1 shows results of NMF treatment on incorporation of formate into combined nucleic acids (Na, counted as sodium salt), glycogen (glyc.), and protein (prot.) of rat liver at 2 hours, 6 hours and 24 hours after administration of isotope. Since all experimental groups had received NMF 48 hours before sacrifice, variations in levels at different time intervals may reasonably be attributed to differences in incorporation of formate at different time intervals after administration. In both nucleic acids and proteins, both control and experimental, lowest levels of incorporation are found at 6 hour interval after administration of isotope.

The level of incorporation of formate into glycogen 2 hours and 6 hours after administration was too low to be of significance in the controls, although NMF apparently increased incorporation or storage of formate in glycogen at these time intervals. However, one cannot rule out the possibility that NMF has no effect on actual rate of utilization of formate for glycogen synthesis, since higher activity may be due to lowered amount of glycogen present. In both the 2 hour and 6 hour experiment we isolated from NMF-treated livers about 40-50% of amount of glycogen isolated from controls, and in the 24 hour experiment about 20% of amount isolated from controls was obtained. Although this was not done quantitatively, the procedures were the same and the livers approximately equal in weight.

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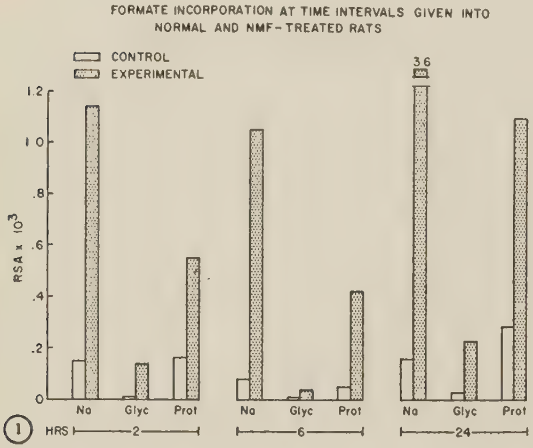
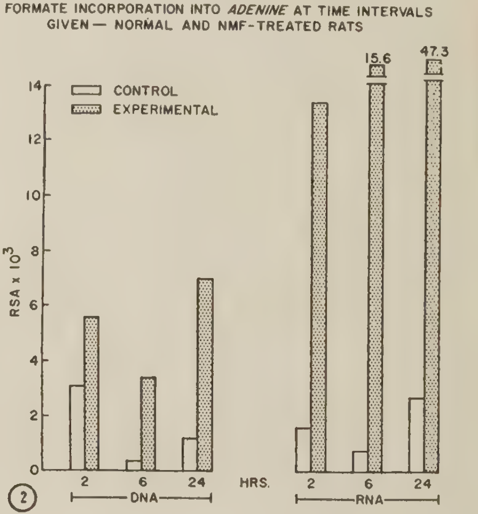
* From Rohm and Haas Co., Philadelphia, Pa.

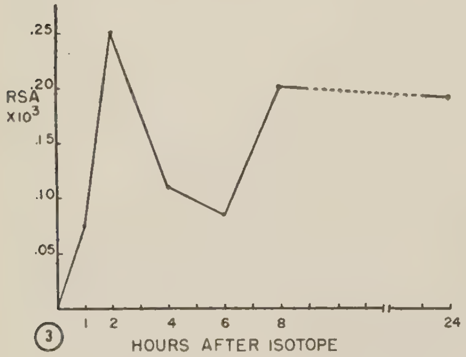
† Obtained from Technical Assn., Burbank, Calif. Diluted with sodium formate to give specific activity of 1 μc/μM.

Fig. 2 gives comparisons of isolated adenine of the DNA and RNA. The striking NMF effect previously reported(1,2) is evident at all 3 time intervals, and it is obvious that the results 2 and 6 hours after injection of isotope are no more informative in regard to effect of NMF, than the results 24 hours after injection of isotope. The results with isolated guanine were qualitatively identical.

In controls specific activity 6 hours after isotope is significantly lower than either the 2 hour interval or the 24 hour interval, just as







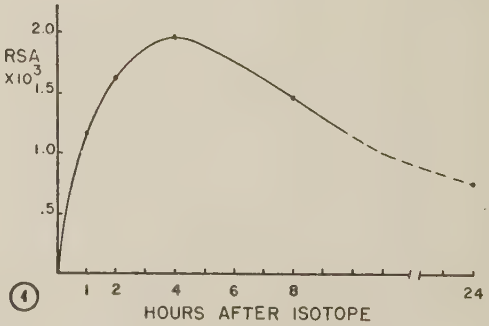


FIG. 1. Formate incorporation at time intervals given into normal and NMF-treated rat livers.

FIG. 2. Formate incorporation at time intervals given into rat liver adenine of normal and NMF-treated animals.

FIG. 3. Formate incorporation at time intervals noted into nucleic acids of normal rat liver.

FIG. 4. Formate incorporation at time intervals noted into nucleic acids of normal rat intestine.

is the case in unfractionated nucleic acids (Fig. 1).

These results prompted us to do the following experiment to see if this was a real effect: 12 rats were each given 4 μc C^{14}-formate; 2 rats were sacrificed at 1 hour, 2, 4, 6, 8, and 24 hours after administration of isotope. Nucleic acids were isolated from combined livers and intestines as described, redissolved in hot 5% NaCl and reprecipitated with ethanol, and counted. The results in liver nucleic acids are graphed in Fig. 3, and in intestinal nucleic acids in Fig. 4.

In the liver, initial incorporation of formate into nucleic acids reached its highest level at 2 hours after injection, with a second peak at 8 hours. The lower RSA at 6 hours than at 2 hours confirms the observations presented in Figs. 1 and 2, although the actual value for 2 hour incorporation was slightly higher in experiment in Fig. 3 (0.25 compared to 0.17 in Fig. 1). The RSA values for 6 hour samples agreed almost exactly (0.1 and 0.09).

The pattern of multiple peaks in the incorporation curve is reminiscent of results presented by Hammarsten *et al.*(5), although the conditions of incorporation which they used are not at all comparable. These investigators injected N^{15}-glycine 5 and 4 hours before sacrifice at various time intervals after hepatectomy. Since the time interval from isotope injection to death was constant, they were measuring relative uptake of N^{15} into purines at progressive stages of liver regeneration. They observed 2 peaks of incorporation: 14 hours and 26-52 hours into RNA, and 26-32 hours and 56 hours into DNA. The double peaks may be due, as Hammarsten pointed out, to the complex heterogeneity of the nucleic acids.

Incorporation of formate into intestinal nucleic acids shows a peak of incorporation 4 hours after administration. There are not enough points after that to know whether there is a second peak after the 8 hour value (the 6 hour sample was inadvertently lost). However, it is of interest (and unexplainable at present) that in the same animals liver nucleic acids show this multi-peak effect but intestinal nucleic acids do not.

It seems, therefore, that choosing a time interval after administration of isotope is largely up to the purpose of the investigator. Two main points to consider in comparing the effect of a drug to control values in experiment of this type are: is action of drug discernible at particular time of examination; and is level of incorporation of isotope high enough to give significant counts at this time? In the present case, it had previously been determined that NMF action is present to a high degree at 48 hours(1). The interpretation of this effect remains the same(1,2) whether the isotope is available to the system for 2, 6, or 24 hours.

Summary. 1) The effect of NMF on rat liver metabolism has been shown to be qualitatively the same at 2 and 6 hour intervals after administration of the isotope as at 24 hours after administration. 2) The first peak of incorporation of formate into rat liver nucleic acids occurs 2 hours after administration, with a second peak 8 hours or after. Peak of incorporation into intestinal nucleic acids is around 4 hours after administration.

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Blocking Antibodies in Rabbits Infected with *Brucella melitensis*.* (24965)

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Blocking antibodies are thermostable antibodies which combine with their specific antigens without visible evidence of agglutination or precipitation. The blocking phenomenon has been observed in insulin resistance, hemolysins, diphtheria toxoid immunization, large injections of pollens and other protein extracts, and the Rh antigen-antibody system. Blocking antibodies also have been reported in association with influenza virus infections and in brucellosis(1). We wish to report a preliminary study on production of blocking antibodies in a small series of rabbits infected with a massive dose of *Brucella melitensis* and the suppressive effect of early treatment with antibiotics.

Method. 1. After blood was obtained for control studies, 6 two-month-old male albino rabbits received *via* ear vein 2 ml of heavy suspension of *Br. melitensis,* strain 363 (courtesy of Dr. W. W. Spink, Univ. of Minnesota Hospitals), grown aerobically 48 hours on tryptose agar. Viable counts of infecting suspension approximated 110 x 10^{14} organisms/ml. Approximately 12 hours after inoculation, 3 of the 6 rabbits were treated with 500 mg streptomycin and 60 mg tetracycline, administered subcutaneously every day for 14 days. The remaining 3 rabbits were left untreated. 2. Blood was obtained at appropriate intervals from all 6 animals *via* ear vein by insertion of a disposable 20 gauge needle. Titers for agglutinating and blocking antibodies, electrophoretic separation of serum proteins and culture for Brucella organisms were obtained from each blood specimen. Agglutinin titer was determined by modification of method of Fitch, Donham, Bishop and Boyd(2). Serum was diluted 2-fold serially from 1:10 to 1:5,120 with 0.85% saline solution. *Br. abortus* antigen (0.5 ml) was added to 0.5 ml of saline (control) and 0.5 ml of each dilution of serum in separate tubes. Final dilution of serum was carried thus to 1:10,240. The antigen was prepared by diluting heavy suspension of heat-killed *Br. abortus* cells to 1:100 with saline solution. Stock antigen suspension was obtained from Agri. Research Service, Animal Disease Eradication Branch, U.S. Dept. of Agriculture. After addition of antigen, the content of each tube was mixed by shaking, then incubated for 48 hours in water bath at 37°C. The tubes were then refrigerated at 5°C for 1 hour. They were examined immediately against a black background with mild agitation. Degree of agglutination was recorded as 0, ±, 1+, 2+, 3+, and 4+, depending on size and number of aggregates as well as degree of clearing of supernate. Agglutinin titer was taken to be the highest dilution of serum showing 1+ or greater agglutination. The blocking test was carried out in same manner as for agglutinin titer, except that 0.03 ml of rabbit anti-brucella serum (agglutinin titer of 1:320) was added to each tube and a saline control tube was included, containing no blocking serum. Blocking was indicated by less agglutination than that in control tube. 3. Electrophoretic studies were carried out in electrophoretic cell (Spinco) using 0.087 M. veronal buffer at pH 8.6. Twenty cu mu of serum was allowed to migrate 16 hours on Whatman No. 3 filter paper. The strips were stained with bromphenol blue dye dissolved in alcohol containing 1% mercuric chloride and then evaluated with Photovolt densitometer (red filter). The areas under individual protein peaks were determined by planimetry. 4. In absorption studies, sera were absorbed twice with heat-killed *Br. abortus* antigen. Each absorption was carried out in water bath at 37°C for 48 hours. 5. Autopsies were performed on all rabbits. Liver and splenic tissues were obtained for histological and cultural studies.

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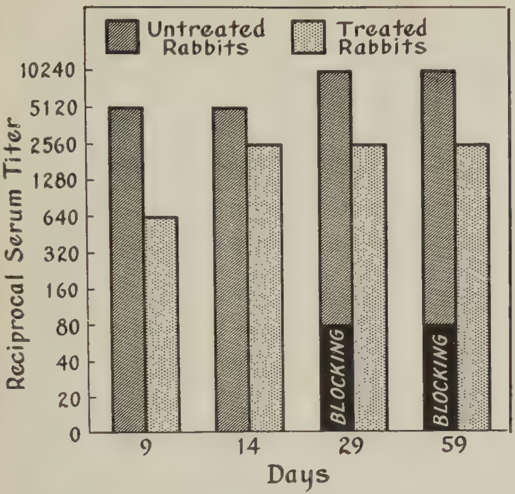

FIG. 1. Antibody titers in sera of rabbits infected with *Br. melitensis.* Blocking antibodies appeared in sera of untreated animals. Prompt treatment with antibiotics seemed to prevent their appearance.

The tissue was prepared for 3 minutes in 50 ml Servall tissue grinder. Aliquot amounts of resulting suspension were cultured on tryptose agar, so that a rough comparison of number of organisms was obtained.

Results. Blood cultures. All control blood cultures were sterile on tryptose agar plates. All infected animals had positive blood cultures within 24 hours after inoculation. In spite of what was considered adequate antibiotic therapy, all treated group of rabbits had persistent Brucella bacteremia for 5 days after initiation of treatment. The untreated group of rabbits, however, suffered persistent Brucella bacteremia for 12 days. One rabbit from untreated group died on 14th day.

Agglutination and blocking antibody studies. Serological studies for agglutinating and blocking antibodies were negative in control sera. Treated rabbits developed a slightly lower agglutinating antibody titer than untreated animals (Fig. 1). Treated animals failed to develop blocking titers throughout the study (59 days), whereas untreated animals (2 surviving) developed a blocking titer of 1:40 and 1:80 respectively by 29th day; this persisted through 59th day without change.

Electrophoretic studies indicated that rabbits left untreated showed a large gamma globulin peak in their sera from 9th day of infection. Gamma globulin levels had increased 2- to 3-fold over those in normal rabbits. Gamma globulin could not be removed by absorption with *Br. abortus* antigen. The treated Brucella-infected animals maintained a relatively normal electrophoretic pattern in their serum during the experiment (Fig. 2, Table I).

Necropsy findings. All untreated animals exhibited a markedly enlarged liver of normal color and consistency. Two had multiple liver abscesses containing greenish-white caseous material; abscesses varied in size from pinpoint to 0.5 cm in diameter. Spleens appeared normal in size and color. No abscesses were found in these organs. Other organs were normal.

Treated animals presented no grossly abnormal findings except for slightly enlarged livers.

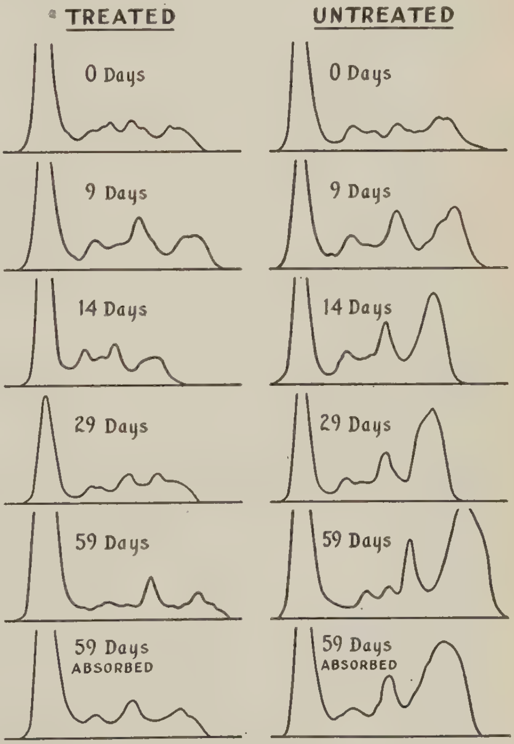

FIG. 2. Electrophoretic patterns of serum proteins of rabbits infected with *Br. melitensis.* Animals treated promptly with streptomycin and tetracycline had normal patterns; those untreated showed a 2-3 fold increase in gamma globulin peak.

TABLE I. Distribution of Serum Proteins in Rabbits Experimentally Infected with *Br. melitensis.* Three animals were treated promptly with a combination of streptomycin and tetracycline, 3 not treated.

Rabbits	Days after infection	Albumin: % of total proteins	Globulins: % of total proteins α-1	α-2	β	γ
Control	0	57.05	4.34	9.83	12.50	16.28
Treated	9	48.21	2.94	9.73	22.88	16.23
	29	53.33	7.19	5.93	15.01	18.54
	59	73.94	3.04	5.17	10.05	7.34
Untreated	9	32.49	2.28	9.91	19.07	35.25
	29	40.09	6.04	4.63	14.78	34.48
	59	45.50	3.50	5.99	13.16	31.92
Absorbed	59					
Treated		63.01	4.02	6.93	13.67	12.37
Untreated		33.80	3.94	9.12	14.56	35.59

Microscopic examination of livers and spleens revealed epithelioid granulomata in untreated animals, compatible with Brucella infections. There also were liver infections with Coccidia. Splenic tissues appeared normal except for one, which showed an abundant proliferation of reticulum with some plasma cells and eosinophils scattered about. Tissues of treated rabbits revealed normal spleens and livers except for one section of liver that showed a possible healing granuloma.

Culture of pooled ground liver and spleen revealed massive Brucella infection only in the liver of untreated animals; only a few colonies of Brucella were cultured from spleens of untreated animals. Cultures from spleens and livers of treated animals yielded only a few colonies of Brucella.

Discussion. These preliminary studies indicated that rabbits infected with large numbers of *Br. melitensis* produced blocking antibodies in low titers by 29th day of infection and persisting through 59th day of observation. In addition, gamma globulins of serum increased 2 to 3 times the normal amount. Absorption of sera with Brucella failed to decrease gamma globulin peak. Rabbits which received antibiotic therapy developed neither increase of gamma globulins nor blocking antibodies.

It appeared that the blocking phenomenon in rabbits infected with Brucella was not correlated with presence of active infection itself, since viable organisms were cultured from both treated and untreated animals; rather, it seems that severity of infection or total amount of antigen in contact with antibody-producing cells might be the deciding factor inducing appearance of the blocking phenomenon. The smaller the amount of active antigen persisting in the animal, the longer it might take to bring about the blocking phenomenon.

This apparent relationship is not unique in brucellosis. Mothers carrying Rh-positive fetuses are known to demonstrate agglutinating anti-Rh antibodies initially, but at a later stage of pregnancy or with subsequent pregnancies by the same father, only incomplete (blocking) antibodies may be demonstrable (3). Experimentally, blocking antibodies induced with purified proteins may appear only after repeated injections with the same antigen(4).

Brahic and others performed experiments similar to ours, inoculating rabbits with *Br. suis.* Using the Coombs test, they were able to demonstrate incomplete antibodies as early as 5 days after infection and lasting more than 60 days(5). Our inability to confirm the early appearance of incomplete (blocking) antibody may depend on the large dose of micro-organisms given, which was about 10^6 larger than that used by Brahic. Furthermore, there was a difference in the test used to demonstrate non-precipitating antibody. Cruickshank infected guinea pigs with *Br. abortus* and concluded as we have, that incomplete antibodies are elaborated only in later stages of infection(6).

The marked increase in serum gamma globulins following untreated Brucella infection has previously been demonstrated experimentally in guinea pigs(6,7) and in man(8,9). Our absorption studies indicate that this gamma globulin is not absorbed by *Br. abortus* cells. This may be indicative of a nonspecific outpouring of proteins in response to infection. There is a possibility, however, that the acute phase gamma globulins contain some nonabsorbable antibody.

Summary. Six rabbits were infected intravenously with a single massive dose of *Br. melitensis.* Three were treated by subcutaneous administration of streptomycin and tetracycline for 2 weeks and 3 were left untreated. The untreated rabbits developed a markedly increased gamma globulin content in their serum, which was not appreciably changed by absorption with killed whole *Br. abortus* cells. In addition, the blocking phenomenon was demonstrated in low titers in serum of these rabbits by 29th day of infection. Antibiotic therapy was effective in preventing the rise of gamma globulins and of blocking antibody through the 59th day.

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A Single-Gene Antagonism Between Mother and Fetus in the Mouse.* (24966)

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Genetic antagonism between mother and fetus has not hitherto been demonstrated in the mouse(2,3). The purpose of the present paper is to demonstrate such a condition. This however differs from the classic types of incompatibility with Rh-antigens in man and erythrocytic antigens in livestock(1), since erythroblastosis fetalis and hemolytic icterus of the newborn has not appeared. A new sort of antagonism is therefore involved. Evidence for fetal antagonism appeared during genetic analysis of a new mutant type named "hair-loss" (Fig. 1). This is a simple recessive Mendelian character which originated as spontaneous mutation in this laboratory. It is characterized by progressive degeneration of hair follicles over entire body, loss of hair becoming obvious after age of about 5 weeks (Fig. 2). There is some resemblance to previously known mutant type, "hairless," gene *hr* in linkage Group III, but the new type, "hair-loss," proves to be in linkage Group VI, and has therefore been given a distinctive gene symbol, *hl*.

Methods. Homozygous hair-loss type has been maintained as a moderately inbred stock. For the present study, outcrosses have also been made with various other genetic types. Linkage-test matings for the VI group involved the marker genes *bt* ("belted"), *Ca* ("Caracul"), and *N* ("Naked"). Reciprocal matings served as controls. Standard mating has been one male with one female, usually not separated for littering. The young were weaned in a different box at about 4 weeks of age. In many cases, litters were recorded

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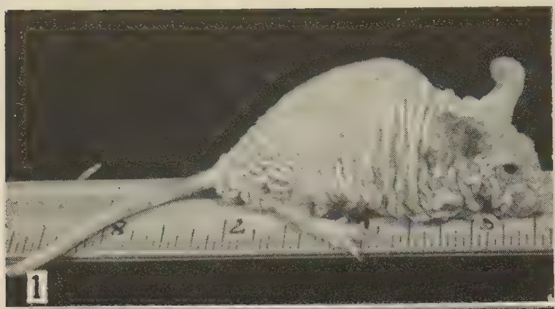

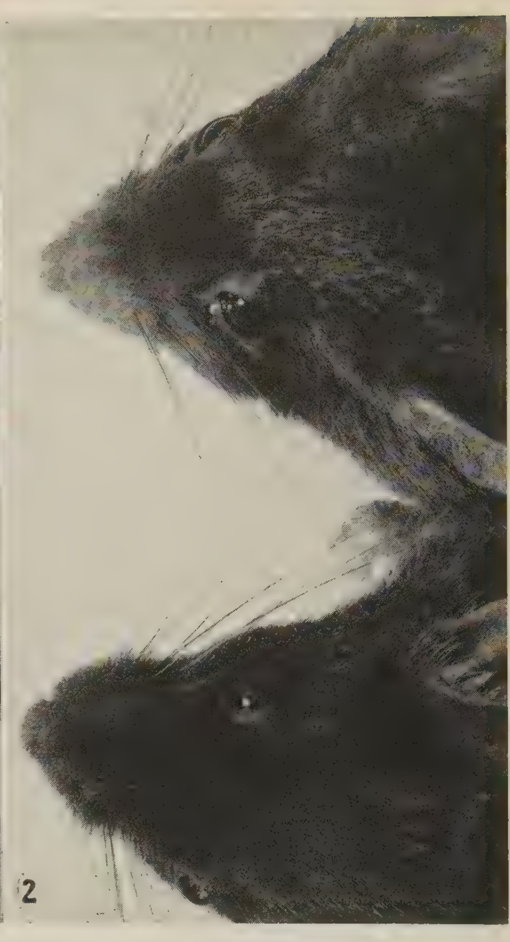

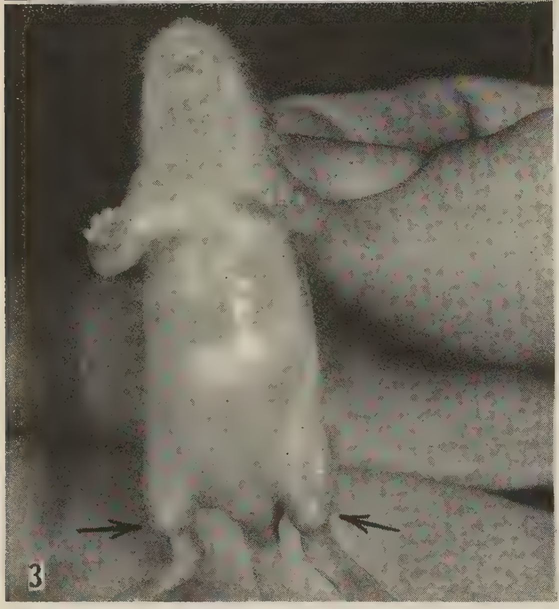

FIG. 1. A mature (7-mo-old) "hair-loss" mouse.
FIG. 2. Five-wk-old mice. Upper: initial stage of "hair-loss." Lower: normal.
FIG. 3. Nine-day-old mouse with spontaneously broken legs (arrows). The mother was "hair-loss."

within 24 hours after birth, and observed daily, but this was not a regular procedure. Classification of marker genes was completed some time between 5 days of age and weaning. Final classification for hair-loss condition was recorded at age of 5 to 8 weeks. Other details of care were routine: weekly changing of boxes by competent personnel, feed† and water *ad lib.* and air-conditioned quarters.

Results. Matings of hair-loss mice to heterozygous normals provided most revealing information. The expected ratio of hair-loss and normal progeny from such matings is 1:1. Where the sire was hair-loss and the dam normal, the results are in very good agreement with this expectation (Table I). However, in the reciprocal mating, where the sire was he-

TABLE I. Reciprocal Matings of Hair-Loss × Heterozygous Normal.

			Progeny classified	
Sire	Dam	Data period	Hair-loss	Normal
Hair-loss	Normal	1.	252	246
"	"	2.	719	700
"	"	3.	168	163
"	"	Total	1139	1109
		Expectation*	1124	1124
Normal	Hair-loss	1.	74	18
"	"	2.	751	440
"	"	3.	267	89
"	"	Total	1092	547
		Expectation*	818.5	818.5

* Genetic ratio 1:1; null hypothesis for mortality effect.

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† Both Purina Lab Chow and Wayne Lab Blox have been used.

TABLE II. Mortality and Survival of Belted (bt) and Caracul (Ca) Progeny in Linkage Tests with Hair-Loss (hl).

Chromosome linkage phase of sire*	Progeny classified: Dying before classification of hair-loss		Progeny classified: Surviving to classification of hair-loss	
	Ca	+†	Ca	+
Ca +	70	59	290	646
+ hl			y 356	
Ca hl	7	28	135	63
+ +			y	72
	bt	+	bt	+
hl +	48	23	178	385
+ bt			y 207	
hl bt	21	26	204	138
+ +			y	66

* All sires were mated to homozygous multiple recessive females.

† The + symbol represents the normal allelic type.

y = apparent mortality.

terozygous normal and the dam hair-loss, the ratio diverged markedly in favor of hair-loss progeny. This result was obtained in 3 successive periods, chronologically, the ratio averaging 2:1.

The discrepancy was readily traced to differential mortality. In the first place, there was no difference in litter size at birth; 72 litters from heterozygous normal females had a median of 9½ young, while 145 litters from hair-loss females gave the same value. Mortality of young, however, was around 3 times as high for hair-loss dams as for normals, particularly before 2 weeks of age. Since these young could not be classified for hair-loss, the marker genes *Ca* and *bt* were utilized as indicators. Their distributions in coupling *vs.* repulsion phases consistently showed higher mortality in the group expected to be non-hair-loss (Table II). Intercurrent disease such as infantile diarrhea was absent during this period.

Much of the mortality occurred even before genes *Ca* and *bt* could be classified. Breakdown of available data indicates that the very early mortality (first 2 days) is related to reproductive work (litter size and lactation) by the dam. This is true for normal dams as well as hair-loss dams, but in the case of the latter is greatly magnified. Litter size shows little relation to mortality where the dam was nursing a previous litter during pregnancy, but in the absence of lactation there does appear to be some correlation of mortality and litter size (Table III). Multiparous females which had not been nursing had a low amount of early mortality.

After age of 2 weeks mortality largely ceased. Examination of the young in high-mortality classes revealed that growth disturbance was the rule. They were generally smaller than their litter mates, and often runty. Commonly between fifth and tenth days of age one or more legs became broken, with swelling and inflammation at the site, generally near middle of tibia and fibula or ulna and radius (Fig. 3). Mice surviving such injury recovered quickly and in no case were of the hair-loss type.

TABLE III. Ratios of Dead/Surviving Young, for the First Two Days after Birth, from Matings of Hair-loss Mice.

Parents: Type of sire	Parents: Type of dam	Nursing while pregnant	Litter-size classes (inclusive): 1–5	6–8	9–11	12–16	Total	Mortality, %
Hair-loss	Normal (hetero.)	No	0/16	0/ 92	6/185	1/ 86	7/379	2
”	” ”	Yes	0/ 5	5/ 38	18/101	4/ 74	27/218	11
”	Normal (homo.)	No					2/217	1
”	” ”	Yes					20/ 56	26
”	Hair-loss	No					0/ 86	0
”	”	Yes					4/ 13	24
Normal (hetero.)	Hair-loss	No	0/21	27/174	47/257	50/175	124/627	17
” ”	”	Yes	18/16	33/ 91	85/119	111/164	247/390	39
Normal (homo.)	”	No					9/116	7
” ”	”	Yes					22/ 33	40

When hair-loss females were mated with homozygous normal males, practically all surviving young suffered the same sort of growth handicap.

Since all evidence pointed to an antagonism of hair-loss mothers toward their non-hair-loss young, the next question was whether the milk was the seat of the disturbance. To test this possibility, newborn young from hair-loss mothers were immediately transferred to normal females for fostering. Here again cases of inferior growth, broken legs, and mortality occurred. Reciprocal fostering was not tried.

Detailed pathological study of young dying in the first few days has not been undertaken. Preliminary observations revealed no significant anomaly; the blood picture seems normal. Death apparently is the consequence of respiratory difficulty, the lungs often being poorly inflated.

Discussion. Minor maternal effects on fetal development in the mouse have previously been demonstrated(2) by reciprocal crosses. Such effects were apparently not associated with known genes, and were not of such antagonistic sort as in the present case. A more striking difference between reciprocal crosses was early noted by Reed(5) for the gene "Fused" (tail vertebrae). However, antagonism was not the mechanism there(3).

In the case of hair-loss, the antagonism shows a close analogy to that involving blood-type genes, in that the mother is of the homozygous recessive type and the affected fetus is heterozygous for the dominant allele. It will be of interest to determine whether the "hairless" (***hr***) type behaves like "hair-loss" in this respect; hairless females have been considered "poor mothers," and not extensively bred(3). Also a comparison with the effects of several "histocompatibility" genes (3) seems needed. Since our mice have been considerably mongrelized for these linkage studies, they probably have a rather random assortment of such genes. However, for this linkage group (*VI*) no histocompatibility locus has been identified.

The mechanism of the antagonism remains to be elucidated. Since there is no evident icterus or red-cell aberration, serological antagonism, if any, probably involves other tissues. No "build-up" of antagonism in multiparous mothers was noted, provided they were not nursing a previous litter during pregnancy. The relation of maternal "over-work" to increased early mortality is most interesting and can account for much variation in data. Possibly the better nutritional status of the young from "rested" dams makes them more able to withstand the antagonism.

Possibly this new sort of antagonism is more related to such incompatibilities as those observed in grafting of plants. Another strong example is a dominant gene, in chromosome III of Drosophila, which causes death of all daughters of a female carrying it, but not the daughters of a male carrying it(4). Conceivably some such principles operate in mongolism and failure of species hybridization.

Summary. A new type of maternal-fetal incompatibility, not involving erythroblastosis, is demonstrated by reciprocal matings in mice. The conditioning factor is a recessive mutant gene named "hair-loss." Normal-hair progeny of hair-loss mothers are ordinarily born alive but suffer excessive mortality during first 2 weeks, even when fostered by normal mothers. The syndrome consists of inadequate lung inflation, inferior growth, and fragile bones. Mortality is very high when a previous litter was being raised by the mother during pregnancy, but seems otherwise not related to parity.

The authors are indebted to Dr. W. J. Miller, Serology Laboratory, Univ. of California at Davis, for information on incompatibility situations in livestock.

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A Suitable Preparation for Pharmacological Analysis of EEG "Activation".* (24967)

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Electroencephalograms characterized by low voltage and high frequency (so-called EEG "activation") can be elicited by stimulation of brain stem reticular formation(1-5). This response often is accompanied by behavioral manifestations of wakefulness. In contrast, a persistent "deactivated" pattern consisting of high voltage, low frequency cortical electrical activity (and behavior which is characteristic of sleep) may be observed following brain stem lesions which destroy portions of mid-brain reticular formation, but which spare the leminiscal fillets; conversely interruption of the primary sensory pathways at mid-brain level without damage to the more medially placed reticular formation does not significantly modify alerting responses and EEG "activation"(4). Observations of EEG effects of diverse pharmacological agents have indicated that mid-brain reticular formation is an important site of drug action and have suggested that multineuronal, polysynaptic character of this area may play an important role in its response to drugs(6-17). Some studies have provided evidence of cholinergic mediation in the "reticular activating system"(12-14). Others demonstrated EEG "activation" following intravenous administration of adrenergic agents, and have suggested that the "reticular activating system" or a component within it is adrenergic(15-17). The anatomical location and functional relation between cholinergic and adrenergic components of the "reticular activating system" have not been clarified. The available data bearing on this problem contain discrepancies which may be due in part to species variation (some investigators using cats, other rabbits). In addition, there has been wide variation in type of preparation used to provide a "deactivated" EEG pattern as baseline for drug-induced "activation" studies. A consistent, reliable and uniform baseline is essential for analysis of unmistakable activation effects. Rothballer(17) stated the problem succinctly: "Whether or not any EEG changes could be detected after intravenous adrenaline depends primarily upon the background of activity, and to a much lesser extent on the dosage. Working against a background of "arousal," it was quite difficult to detect any effect at all." Our experience has substantiated this view, and brought to our attention certain disadvantages of some preparations commonly used. In the unanesthetized curarized cat, artificial respiration is necessary, often leading to disturbances in EEG pattern; external stimulation (noise, light, handling) must be reduced to extremely low levels, and even then a reliable "deactivated" EEG background is difficult to reproduce consistently. These difficulties can be overcome with the "cerveau-isolé"(20), but like other preparations in which the cranium is widely opened, cerebral edema generally develops. Moreover, the "cerveau-isolé" suffers from a tendency towards progressive cardiovascular deterioration, and lacks an intact brain stem. The cat with midbrain reticular coagulation(17) exhibits a good "deactivated" EEG pattern as well as reliable cardiovascular status, but here again the brain stem is not intact and coagulative lesions may eliminate important sites of drug-induced effects. Moreover, residual effects of anesthesia in acute preparations may be difficult to exclude. A "deactivated" pattern follows intra-cranial destruction of trigeminal nerves in the "encephale isolé" cat. Electrolytic transection of mid-brain at the rostro-pontine level (rostral to the trigeminal roots) produces a similar result(18-19). It is generally believed that physiological "activation" of EEG is dependent upon the play of impulses from collateral fibers feeding into the reticular formation. Our objective has been to produce a sufficient degree of somatic de-afferentation in an unanesthetized preparation with intact brain stem

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* This investigation was supported by Federal Mental Health Grant.

so as to elicit a consistently high degree of EEG "deactivation" without the aid of extraneous pharmacological agents. Such a preparation should also exhibit good cardiovascular status and should not require artificial respiration.

Methods. A preparation which meets the above desiderata can be accomplished by extra-cranial transection of the roots of maxillary and mandibular branches of trigeminal nerves followed by low transection of cervical spinal cord and sensory roots of cervical spinal nerves. 1. *Extra-cranial transection of trigeminal roots.* Cats anesthetized with pentobarbital sodium (35 mg/kg) are placed in the inverted position in a stereotaxic holder. The lower jaw is opened maximally to expose the roof of mouth. An incision 1.5-2.0 cm long is made 2 mm lateral to the edge of the pterygoid process of the sphenoid and the perpendicular plate of the palatine. Adequate exposure is achieved by careful separation and removal of the external and internal pterygoid muscles, and the maxillary and mandibular roots of trigeminal nerves are sectioned bilaterally at their points of emergence from the foramina ovale and rotundum. The fossae created by removal of pterygoid muscles are packed with sterile gelatin sponge and the incision sutured. In large cats (4-5 kg), cord transection can be done immediately following partial "trigeminalectomy," but in smaller cats this is done after 12 to 24 hour rest period. 2. *Low transection of cervical spinal cord and cervical sensory roots.* The surgical technic for cord transection is essentially similar to that described by Bremer(20), but with the following elaboration. The dorso-lateral surface of the atlas and the axis, and spinous processes and dorsal vertebral arches of C_3 to C_7 are exposed. The atlantal foramina and the intervertebral foramina between axis and atlas are located bilaterally and the dorsal roots of C_1 and C_2 cautiously sectioned to avoid bleeding from accompanying blood vessels. Dorsal arches and spinous processes of remaining cervical vertebrae are removed and their cut ends packed with sterile bone wax. With a dural hook, the dura at level of C_7 is then elevated and a longitudinal incision made rostrally to C_2. The dorsal roots of C_3 to C_8 are gently elevated and sectioned bilaterally after high frequency coagulation of dorsal radicular arteries that accompany these roots. The dorsal spinal artery is then coagulated just rostral to the point chosen for cord transection. The cord is then severed between last cervical and first thoracic vertebrae and the section is further explored by gentle suction to make sure that all connecting strands of cord tissue are separated. Sterile gelatin sponge is placed between sectioned ends of the cord, the dura sutured and the incision closed.

Results. This preparation recovers readily from spinal shock, and may be maintained 2 to 3 months if suitable precautions are observed and adequate nursing care provided. This care includes antibiotic administration when necessary, maintenance of reasonably uniform and physiological environmental temperature, bladder catheterization and feeding by stomach tube. Such preparations maintain spontaneous diaphragmatic respiration (24-30/min.) and blood pressures of 85-100 mm Hg. Although some degree of motor innervation to muscles of neck and forelimbs remains intact, the animal generally remains quiescent. Sensory inflow is limited to special and visceral afferents of cranial nerves, as well as somatic afferents to orbit and upper eyelid *via* the ophthalmic branch of trigeminal nerve. The eyes are able to follow an object passing through the visual field and react with pupillary changes to emotional stimuli.

All observations reported below have been repeated a number of times in several different preparations. With this preparation, it has been possible to carry out all of following procedures in the unanesthetized non-curarized cat. (a) To insert an indwelling polyethylene catheter *via* the right subclavian artery so that its tip lies at the bifurcation of innominate artery. With this technic intracarotid administration of drugs has been carried out without cannulation of these arteries. Bilateral and simultaneous delivery of drugs to both sides of brain has been accomplished and direct drug-induced effects on EEG observed without appreciable latency. (b) To place the animal in a stereo-taxic instrument without eliciting motor activity or EEG dis-

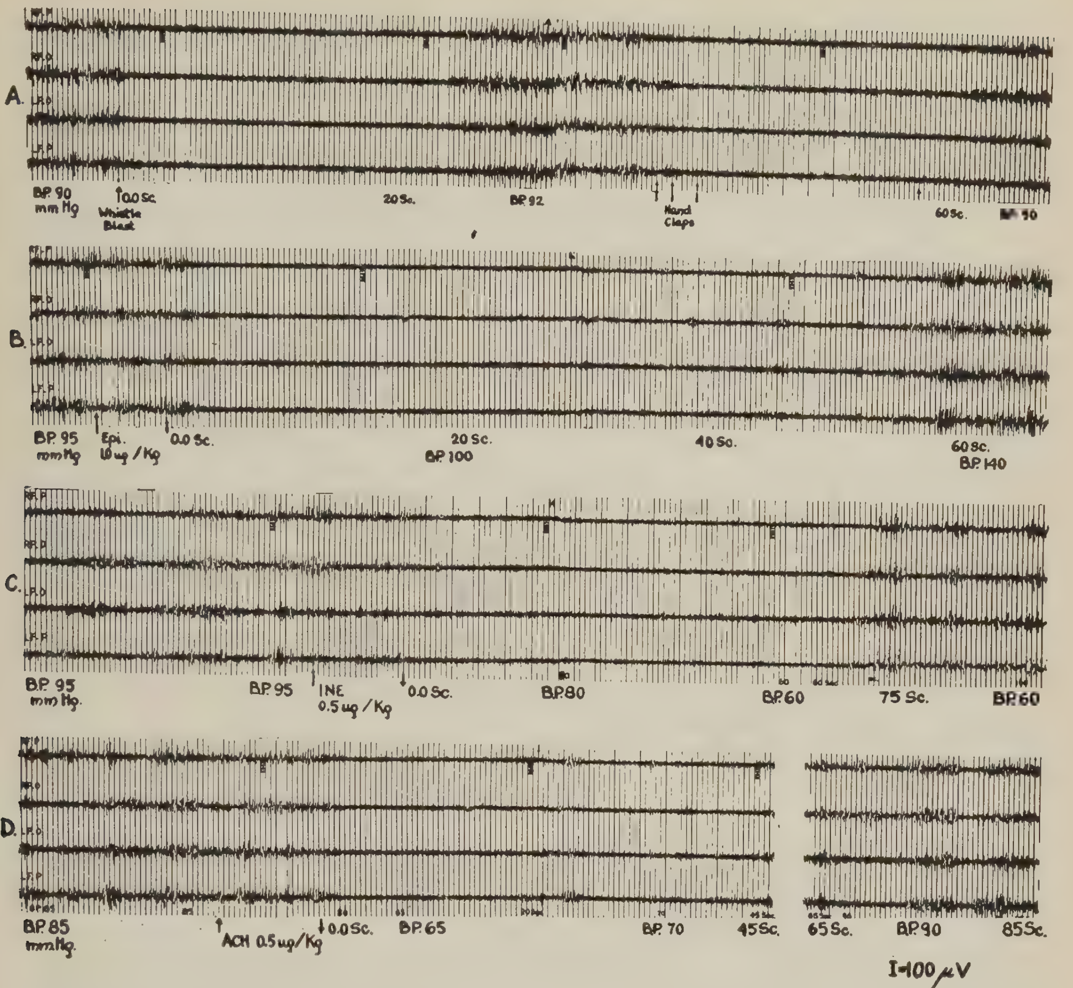

FIG. 1. "Activation" effects of auditory stimuli and of adrenergic and cholinergic agents administered by intra-innominate route in a de-afferented, unanesthetized, non-curarized cat with intact brain stem. All doses expressed as weight of free base. A: Audiogenic activation (whistle blast and hand claps). Prompt onset in both instances with abrupt reversion to the pre-activated pattern after 15-20 sec. B: Intra-innominate epinephrine (1 μg/kg). Injection bracketed by arrows. Immediate activation preceding pressor response (latency = approx. 3 sec.), and lasting approximately 57 sec. with abrupt reversion to control pattern while the pressor response is maximal (140 mm Hg). C: Intra-innominate isopropyl norepinephrine (0.5 μg/kg). Injection bracketed by arrows. Immediate activation preceding peak depressor response and lasting about 75 sec., with abrupt reversion to pre-existing pattern when the B.P. is still at 60 mm Hg. D: Intra-innominate acetylcholine (0.5 μg/kg). Injection bracketed by arrows. Immediate activation before peak depressor response (65 mm Hg), with a gradual return to pre-activated pattern.

turbances. (c) To expose the cranium and place screw electrodes through the skull with tips touching the dura for recording electrocorticograms. (d) To trephine the skull and insert tips of coagulating electrodes into the brain stem for production of electrolytic lesions. In such preparations, an indwelling arterial catheter has been maintained for several days so that the effects of a number of agents, even long-acting compounds, could be observed in the same animal. The importance of durable chronic preparations which can be studied after ample time for recovery from destructive lesions of the central nervous system has been stressed recently by Batsel *et al.* (21). Furthermore, the initially intact midbrain stem makes it possible for one side to act as a reliable control for drug-induced EEG effects, following mid-brain lesions of the contralateral side.

This preparation has provided a particularly useful tool for study of drug-induced "activation" of EEG, inasmuch as a highly deactivated EEG can readily be produced in absence of brain stem lesions and extraneous drug influences. In addition, cerebral edema is lacking and cortical excitability is good. After a few days of adaptation to its new situation, a Rothballer(17) type "D" pattern (persistent spindles in all leads) regularly appears when the animal is placed in a quiet, dimly lighted environment, even in the stereotaxic instrument. Low-voltage, fast frequency responses are usually observed during feeding. Marked and immediate EEG "activation" can be accomplished easily by suitable auditory (or visual) stimulation (Fig. 1A), but prompt reversion to a "deactivated" pattern follows removal of the stimulus. Moreover, this preparation manifests a consistently high degree of sensitivity to direct (intra-arterial) "activation" by both cholinergic and adrenergic agents (Fig. 1B, C, D).

Preliminary results with this preparation indicate that transitory drug-induced EEG "activation" immediate in onset, and with complete temporal independence of blood pressure, occurs at relatively low dose levels (0.25-1 μg/kg) following intra-innominate administration of epinephrine, norepinephrine, isopropyl norepinephrine and acetylcholine.

Summary. (1) A method is described for obtaining a suitably de-afferented preparation with intact brain stem in the unanesthetized cat whose resting EEG pattern is well "deactivated" and ideally suited to pharmacological and neurosurgical analysis of adrenergic and cholinergic components of the "reticular activating system," free from extraneous pharmacological influences. (2) Preliminary observations with both adrenergic and cholinergic agents suggest that complete temporal independence can be shown between onset and termination of action of "activating" agents introduced directly by intra-innominate administration and vascular effects of these agents as reflected in blood pressure alterations. (3) Advantages of this preparation over those commonly used for drug-induced EEG "activation" are reported. (4) This method currently is being used for further anatomical exploration of adrenergic and cholinergic components of pathways leading to diffuse "activation" of the electro-corticogram, and for studying the influence of psycho-tropic drugs on these systems.

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Failure of Cell-Free Spleen Extracts to Protect Irradiated Animals.* (24968)

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It is a current hypothesis that effectiveness of postradiation treatment with hematopoietic containing tissues is a function of "cellular repopulation" and/or "transitory activity" of injected cells pending recovery of recipient marrow rather than any elusive "humoral" factor which stimulates recovery. Evidence presented by Ford *et al.*(1), Odell *et al.*(2), Makinodan(3), Smith *et al.*(4), and Nowell *et al.*(5), has demonstrated the replacement of circulating erythrocytes, platelets and leukocytes in x-irradiated recipients by parenterally introduced cellular components from non-irradiated donors. Jacobson(6) has reported that at least 50,000 spleen cells are required to enhance survival of irradiated mice. On the other hand, Ellinger has reported protection of mice(7) and guinea pigs(8) against death following radiation by injection of a cell-free mouse spleen extract. In this report the results are given of experiments conducted to determine the value of cell-free mouse spleen extracts employed as a protective agent for mice and guinea pigs exposed to whole body x-irradiation.

Materials and methods. (a) Spleens from 350 normal albino mice (*Mus musculus*) of mixed gender, age and size were removed following ether anesthesia. The spleens were placed in a precooled mechanical blender containing 350 ml of 0.85% saline and homogenized in 2 periods of 1 minute each with cooling in between. The homogenate was alternately frozen and thawed 3 times for maximum cellular disruption. The mixture was then placed in a refrigerator for 24 hours after which it was centrifuged in a refrigerated centrifuge at high speed. The supernate was passed through several layers of Whatman No. 1 filter paper in a Buchner funnel under light negative pressure. Microscopic examination of numerous fields of the resulting filtrate failed to reveal any intact cells, therefore the extract was assumed to be cell-free. The extract was then lyophilized and stored at –20°C. The entire procedure was conducted under aseptic conditions at low temperatures. Forty-five mice similar to those used for preparation of the splenic extract were exposed to approximately 600 r total body x-irradiation by means of a Westinghouse Quadrocondex x-ray machine. The radiation factors were: 250 KVP; 15 ma., 1.0 mm Al and 0.5 mm Cu filters in addition to an inherent filtration of 2.5 mm Al and 0.25 mm Cu; size of field, whole-body; target distance, 16 inches. The mice were irradiated for 6.7 minutes at approximately 90 r/min in groups of 9 in a cylindrical cardboard container 17 cm in diameter and 3 cm high. The dosage corresponded to a $LD_{100}/10$ days. The mice were randomly divided into 2 groups of 20 and 25; the larger group served as controls. The lyophilized spleen extract was resuspended in 0.85% sterile saline to give a total volume of 1 ml per 4 spleens. This extract was injected intraperitoneally, 0.1 ml immediately following exposure the day of radiation and 0.6 ml administered at a dose of 0.2 ml per day on the first, second and third post-irradiation days for a sum total of 0.7 ml. (b) Thirty-three guinea pigs of mixed gender, each weighing about 600 g were irradiated for 4.4 minutes at approximately 90 r/min in groups of 4 under the same conditions as the mice, each receiving a total body dose of about 450 r. The radiation intensity in this case, as with the mice, was checked with a Victoreen r meter. This dosage corresponded to a $LD_{85}/10$ days under these conditions. These animals were randomly divided into 2 groups of 20 and 13 each, the smaller group serving as controls. The treated animals were injected intramuscularly with 0.5 ml of the spleen extract immediately after irradiation and 2.0 ml was administered at a dose of 0.5 ml per day given on the first,

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† Public Health Service Research Fellow.

second, third, and fourth post-irradiation days for a sum total of 2.5 ml.

Results. The results of the experiments are shown in Figs. 1 and 2. It can be seen that post-irradiation treatment of whole body x-irradiated mice and guinea pigs with cell-free spleen extracts under the conditions noted, did not result in protection against postradiation mortality. In fact, treated animals died at an accelerated rate when compared to the death rate of controls.

The cell-free spleen extract preparation used was similar to but different from that of Ellinger, and the induced x-irradiation mortality was higher and more rapid with both guinea pigs and mice than in Ellinger's experiments. Irradiated animals died within 10 days whereas in Ellinger's experiments significant differences in mortality rates of spleen-treated animals and their saline controls were not evident prior to the fifteenth day after irradiation. Other significant deviations from Ellinger's technic were reconstitution of lyophilized suspensions to yield injectable material equivalent to 4 spleens rather than 5, the injection of this material for 3 or 4 days after irradiation rather than for 5 days, the use of repeated freezing and thawing of spleen cell suspensions to obtain cell-free extracts and the use of ether anesthesia to sacrifice the animals from which spleen extracts were prepared. Finally, the intraperitoneal rather than intramuscular route was employed for injection of mice although not guinea pigs. It is possible that the differences in preparation and administration of the cell-free extract as well as the method and time of storage may have been destructive to the "humoral spleen factor(s)" and/or that the higher dosage of irradiation was sufficient to mask the effects of the factor(s) if present. Under the experimental conditions employed protective humoral factors could not be shown to exist in cell-free mouse spleen extracts. These results suggest that strict adherence to Ellinger's technic methods is critical.

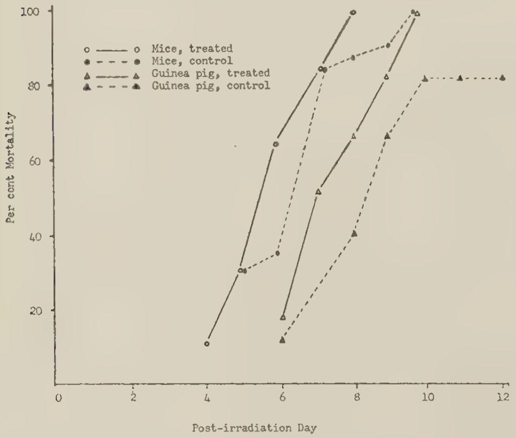

FIG. 1. Mortality of adult albino mice treated intraper. and guinea pigs treated intramusc. with cell-free spleen extract following 600 r and 450 r total body x-irradiation, respectively.

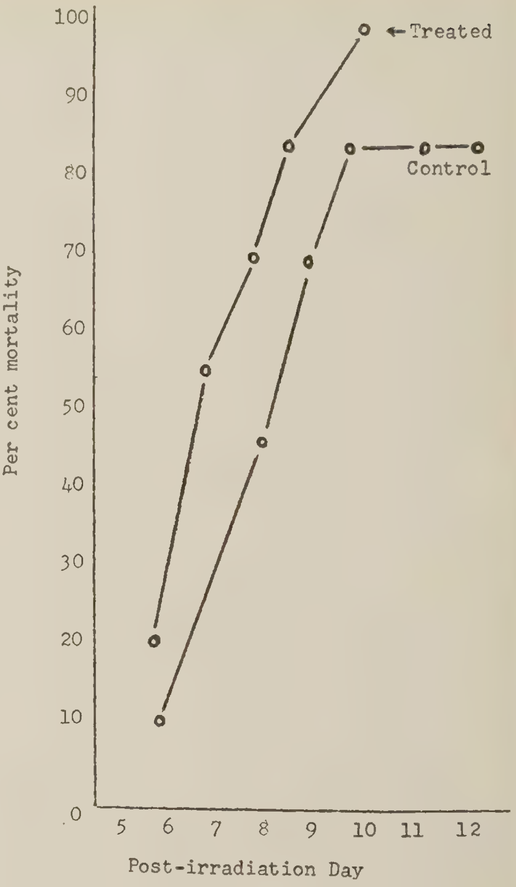

FIG. 2. Mortality of guinea pigs treated intramusc. with cell-free spleen extract following 450 r total body x-irradiation.

Summary. Postradiation administration of cell-free mouse splenic extract to whole body x-irradiated mice (intraperitoneally) and guinea pigs (intramuscularly) did not result in protection against postradiation death.

The treated animals died at an accelerated rate when compared to the controls.

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Some Relationships Between Peripheral Monoamine Oxidase Inhibition and Brain 5-Hydroxytryptamine Levels in Rats.* (24969)

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Recently Sjoerdsma *et al.*(1) suggested *in vivo* assay in man of monoamine oxidase (MAO) inhibitors based on ability to suppress excretion of 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of 5-hydroxytryptamine (5-HT). Since rats excrete 5-HIAA(2), it seemed probable that the Sjoerdsma procedure could be modified into routine method of evaluation of *in vivo* peripheral MAO inhibition in the rat. In addition, by measuring the effect of same MAO inhibitors on brain 5-HT levels a comparison of peripheral with central effects would be obtained.

Methods and materials. 5-HIAA excretion: Male albino rats (175-225 g) were obtained from Carworth Farms. Compounds were administered orally to minimum of 6 rats. At various times after administration of inhibitor, 10 mg/kg of 5-HT (free base) was administered orally. Rats were then placed in metabolism cages and urine collected over subsequent 6 hours. Urines were stored in refrigerator overnight and analyzed next day. The nitroso-naphthal method(3) was employed to measure 5-HIAA levels with following modification: One ml of urine was placed in 50 ml glass-stoppered bottle containing 5 ml of water, to which was added 6 ml of 2,4-dinitrophenyl-hydrazine reagent. Optical measurements were performed with Beckman DU spectrophotometer and Pyrocell microcuvettes. *Brain 5-HT concentrations*: The procedure of Bogdanski *et al.*(4) was employed. 5-HT was determined with Farrand spectrofluorometer. The following test compounds were employed: Phenylisopropylhydrazine: PIH or JB-516 (Dr. J. Biel, Lakeside Labs); 2-isopropyl-1-isonicotinyl hydrazine: IPH or iproniazid (Dr. L. O. Randall, Hoffmann-LaRoche); harmine, harmaline, and tetrahydroharmine (Dr. F. H. Clarke, Schering Corp.); the authors are indebted to these investigators for these compounds.

Results. 5-HIAA excretion: Excretion of 5-HIAA by normal rats is 8.9 γ/100 g BW (95% confidence limits: 7-10.8 based on 18 determinations). Rats given 10 mg/kg of 5-HT orally will excrete 65.8 γ/100 g BW (95% confidence limits: 58.3-73.3 based on 83 determinations) or 5.6% of dose in urine as measurable 5-HIAA over 6 hours. Erspamer(2) reported that the rat excretes 5.5% of orally administered 5-HT (6 mg/kg) as 5-HIAA. Preliminary experiments indicated that inhibitor studies were more reliable when employing the exogenous 5-HT test rather than measuring only the endogenous 5-HIAA.

Fig. 1 indicates efficacy of various substances in diminishing excretion of 5-HIAA. The most potent compound was PIH. Harmine, harmaline, and tetrahydroharmine were somewhat less potent. Lysergic acid diethyl-

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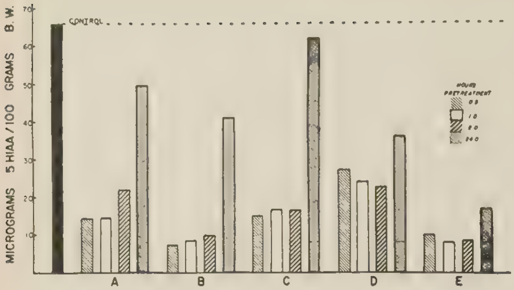

FIG. 1. Effect of harmine (A), harmaline (B), tetrahydroharmine (C), iproniazid (D), and JB-516 (E) on 6 hr excretion of 5-hydroxyindoleacetic acid in urine. Inhibitors (10 mg/kg) administered orally at times indicated prior to administering 10 mg/kg 5-hydroxytryptamine. Control excretion was 65.8 γ/100 g BW (95% confidence limits: 58.3-73.3).

amide and D-amphetamine (10 mg/kg PO), both of which are weak MAO inhibitors, *in vitro,* reduced 5-HIAA excretion from 65.8 γ to 53.6 and 57.1 γ/100 g BW respectively. Pentobarbital (10 and 35 mg/kg PO) was inactive as inhibitor of 5-HIAA excretion.

When doses of IPH less than 5 mg/kg were administered 0.5 hour prior to 5-HT, IPH was ineffective in decreasing excretion of 5-HIAA. A single dose of 5 or 10 mg/kg, however, reduced excretion of 5-HIAA from control level of 65.8 γ to 49.1 and 28.6 γ/100 g BW, respectively.

5-HT brain levels. Harmine, unlike PIH or IPH, did not markedly raise the level of 5-HT in brain when administered orally (Table I). However, when these drugs were administered by intraperitoneal route, harmine was as active as PIH and more active than IPH (Table I).

Discussion. The 5-HIAA excretion test appears to be sensitive and may be employed to evaluate MAO inhibitors. The most potent compound was PIH. Twenty-four hours after administration of PIH, administration of 5-HT was not followed by usual rise of 5-HIAA excretion in urine. No other agent tested was as effective as PIH. The potency of PIH agrees with that reported by Horita (5) and Biel *et al.*(6) employing other technics.

Comparison of harmaline, a potent MAO inhibitor(7), with PIH in the 5-HIAA excretion test indicates that these agents were approximately equiactive until the 24th hour. Differences at this time could be explained on the basis of rapid metabolism of harmaline(8) and relatively slow inactivation of PIH. Harmine, harmaline, and tetrahydroharmine appeared to be approximately equiactive.

Increase in 5-HT concentrations in brain after oral administration of PIH and harmine did not parallel the effects noted in the 5-HIAA excretion test. Although PIH was quite potent, harmine was relatively inactive. However, when given by intraperitoneal route, harmine considerably increased rat brain 5-HT concentration. This effect was equivalent to that noted previously with harmaline. It appears, therefore, that ineffectiveness of oral harmine in increasing the levels of brain 5-HT is due to lack of absorption from gastrointestinal tract or a more rapid inactivation when administered by this route.

Since 5-HT usually does not penetrate the "blood-brain barrier"(9), any blockade of 5-HT metabolism as measured in the 5-HIAA test is a manifestation of a predominant peripheral rather than central action. This type of specificity may be useful in broad evaluation of compounds. For example, pentobarbital, which increases brain 5-HT concentrations(10) does not inhibit excretion of 5-HIAA. The 5-HIAA method, therefore, of-

TABLE I. Increase (%) of Rat Brain 5-Hydroxytryptamine* after Administration of Monoamine Oxidase Inhibitors (10 mg/kg).

Hr post Rx	1		2	24
Route	Oral	Intraper.	Oral	Oral
Inhibitor				
JB-516 (PIH)	80 (6)† [71-90]‡	84 (6) [67-100]	110 (6) [100-119]	97 (12) [82-135]
Iproniazid (IPH)	7 (6) [7]	24 (12) [11-39]	23 (6) [19-26]	46 (6) [44-48]
Harmine	9 (6) [8-10]	79 (6) [76-81]	27 (3) [27]	0 (6) [0]

* 5-HT concentrations for normals, 41-77.8 γ/g of brain, mean 54.6.

† Total No. of animals used.

‡ Range of values.

fers a means of detecting MAO inhibition directly, and when used in conjunction with other technics, serves to enlarge the pharmacology of various drugs.

Summary. A method for measuring activity of monoamine oxidase inhibitors as a function of suppression of 5-hydroxy-indoleacetic acid (5-HIAA) excretion is described. Increased excretion of 5-HIAA after 5-hydroxytyramine (5-HT) is effectively blocked by orally administered iproniazid, JB-516, and several harmala alkaloids. Correlation of these results with increased brain 5-HT is presented. Instances of non-correlation such as lack of effect of oral harmine on brain 5-HT with concomitant suppression of 5-HIAA excretion are discussed.

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Induction of Plasma-Cell Neoplasms and Fibrosarcomas in BALB/c Mice Carrying Diffusion Chambers. (24970)

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Plasma-cell neoplasms are rare in mice (1,2). Rask-Nielsen(2) increased the incidence of these tumors in mice of 2 strains by injecting a carcinogen into the thymus. The findings reported here suggest that a new method of inducing plasma-cell tumors has been found. Seven plasma-cell tumors arose in mice in 2 experiments which were set up by Dr. Algire for another purpose. The tumors arose in BALB/c mice, a strain in which plasma-cell tumors have not been found previously. These induced tumors are of particular interest because at least 4 are transplantable and synthesize abnormal proteins. Six spindle-cell sarcomas also arose but similar tumors have been found in other experiments in association with diffusion chambers. Experiments are now in progress to obtain more information about induction of plasma-cell tumors, but since it will require a year or more to complete them, preliminary findings are reported now.

Procedure. Diffusion chambers were of the cell-impenetrable type described by Algire(3). The chambers were made of lucite rings and Millipore membranes which had pores averaging 0.10, 0.05 or 0.01 μ diameter. Tissue for placement in the chambers was obtained from spontaneous mammary tumors that arose in C3H mice carrying the milk agent. Two tumors were used, one in each experiment. A piece of tumor tissue about 0.5 mm diameter moistened with balanced saline containing tris buffer, horse serum, penicillin and streptomycin(3), was sealed into each chamber. The chamber was inserted into peritoneal cavity of agent-free BALB/c female mouse 5 or 6 weeks of age. A 5 mg pellet of 5 or 10% diethylstilbestrol in cholesterol was placed subcutaneously when mouse was 2 to 4 months of age. Additional mice in each experiment received intraperitoneal injection of balanced saline containing 0.04 ml of a tissue mince of the mammary tumor tissue. These mice also received stilbestrol.

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*Grateful acknowledgement is made to Dr. Thelma B. Dunn of Dept. of Pathology, Nat. Cancer Inst. for diagnosing the tumors and supplying photomicrographs.

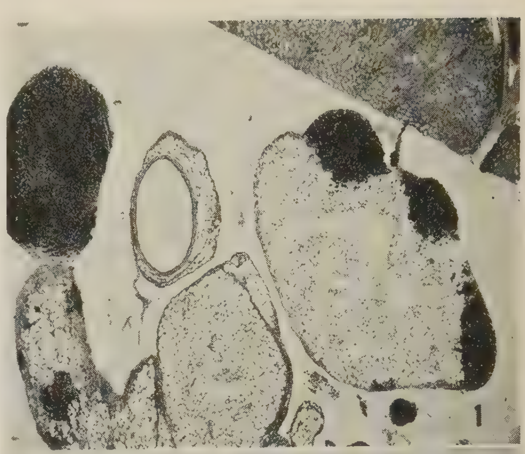
† Deceased.

Results. Forty-six mice carrying diffusion chambers survived 6 months or longer. By 17 months, 7 had developed plasma-cell tumors and 6 had developed sarcomas (Table I). The 7 tumors identified histologically as plasma-cell tumors were inoculated into BALB/c mice. Two have not yet grown at 4 and 22 weeks respectively. Another grew on transfer for one generation but was not transferred again. Four have been successfully transplanted for 2 or more generations. In the first generation at least one tumor in each line grew at site of inoculation within 4 to 11 weeks. One tumor that grew in BALB/c mice, was inoculated at the same time into C3H mice where it failed to grow. Two of 6 sarcomas were transferred to both C3H and BALB/c mice but they grew only in strain BALB/c. A small fibrosarcomatous growth was found on connective tissue coating removed from one diffusion chamber. This chamber was placed in a second host and 2 months later a fibrosarcoma with areas of ossification was found in the abdominal cavity. One or 2 mice in each cage died for reason other than growth of plasma-cell tumors or sarcomas. Three had ascites but no abnormal growths were found. Twenty-one mice are still living. Neither plasma-cell tumors nor sarcomas developed in any of the 38 mice that survived over 6 months after receiving an intraperitoneal injection containing mammary tumor tissue.

TABLE I. Incidence of Plasma Cell Tumors and Sarcomas in Strain BALB/c Mice 6 to 17 Months after Intraperitoneal Implantation of Diffusion Chambers Containing C3H Spontaneous Mammary Tumor Tissue.

Incidence: $\frac{\text{Mice with tumors}}{\text{No. with chambers}}$

	Exp. I		Exp. II	
Diffusion chamber, avg pore size (μ)	Plasma-cell tumors	Sarcomas	Plasma-cell tumors	Sarcomas
.10	1/9	1/9	0/6	1/6
.05	1/8	0/8	1/7	3/7
.01	4/8	1/8	0/6	0/6



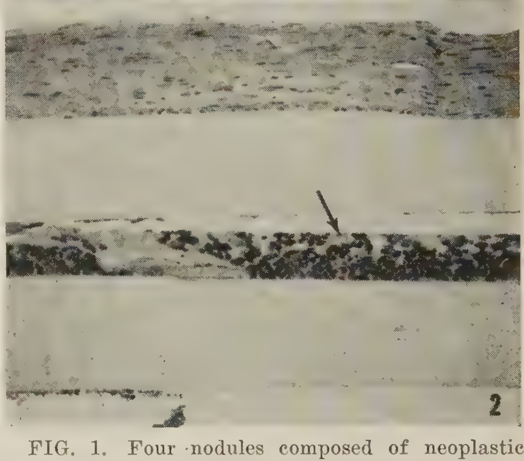


FIG. 1. Four nodules composed of neoplastic plasma cells attached to peritoneal surface where peritoneum covers adipose tissue of mesentery. Hematoxylin and eosin. × 25.

FIG. 2. Portion of cross section of diffusion chamber showing mammary tumor tissue (arrow) between 2 millipore membranes and showing layer of host fibrous tissue on outside surface of one of the membranes. Hematoxylin and eosin. × 105.

Pathology. The 7 mice with plasma-cell tumors had detectable ascites from less than 2 weeks to over 3 weeks at time of autopsy. In 5 of the 7 mice, the ascites was bloody. One side of diffusion chamber was close against the liver in most mice and the other side was coated with thick layer of connective tissue. Small nodules of tissue were distributed over entire peritoneal surface (Fig. 1). Larger nodules of various sizes were found in 5 mice but none were attached to tissue around the chamber. In contrast, in all mice with sarcomas the largest and in some cases the only tumor mass was attached to connective tissue coating around the chamber.

Microscopically the plasma-cell neoplasms were composed of large cells with plasma-cell characteristics. Metastases were frequent. They were found in ovary, pituitary, spleen and lymph nodes. One tumor line caused a myeloma type change in kidney and another line produced osteolytic lesions.

Either whole mounts or sections were made of the porous membrane with adjacent cell layers from some diffusion chambers (Fig. 2). Viable appearing mammary tumor tissue was found in 4 of 5 chambers from mice that developed plasma-cell tumors. Histologically recognizable mammary tumor tissue was present in 3 of the 4 chambers taken from mice with sarcomas. The cells appeared necrotic in one chamber but the tissue surrounding the chamber was also necrotic.

Discussion. Induction of plasma-cell tumors was an unexpected result of these experiments originally set up to study passage of mammary tumor agent through pores of various sizes. The findings on mammary tumors will be published separately.

Unpublished observations show that rings, each with 1 membrane attached, induce sarcomas when implanted subcutaneously, but further experiments are needed to elucidate the role of diffusion chamber parts, the tissue inside the chamber and the stilbestrol in induction of plasma cell tumors.

Summary. Seven plasma-cell neoplasms and 6 fibrosarcomas developed in strain BALB/c mice that carried diffusion chambers in the peritoneal cavity. The diffusion chambers contained mammary tumor tissue obtained from strain C3H mice with the milk agent. Five plasma-cell tumors and all sarcomas were successfully transplanted to strain BALB/c mice. The 2 plasma-cell tumors and the 2 sarcomas transplanted to C3H mice failed to grow.

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Regulation of Cholesterol Oxidation by Liver *in vitro*.*† (24971)

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Careful balance studies of absorption and excretion of bile acids and cholesterol in rats (1) indicated that administration of bile acids in the diet depresses mobilization of liver cholesterol. Further analysis of this phenomenon in both rats(2,3) and mice(4) has shown that both rates of synthesis and mobilization of liver and adrenal cholesterol are reduced upon feeding bile acids. Bile acids are major products of cholesterol catabolism in the liver (5). Daily production of bile acids in the rat is greatly enhanced when enterohepatic circulation of acids is interrupted by cannulation of bile duct(6,7). Bergstrom and Danielsson(8) showed that it is the concentration of bile acid conjugates in portal blood which actually influences rate of further synthesis of bile acids in the liver. These findings indicate that *in vivo,* the level of recirculating bile salts controls cholesterol catabolism in liver and further production of bile acids. We have now observed this "feedback effect" in an *in vitro* system prepared from rat liver, which oxidizes cholesterol to CO_2 and bile acids (5,9).

Methods. Cholesterol-27-C^{14} was synthesized from 3β-hydroxy-5-norcholestene-25-one(10-11) and purified *via* the digitonide

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* This work supported by grant from Nat. Heart Inst., N.I.H., Bethesda, Md.

† We are much indebted to C. Witmer and Drs. J. C. Thompson and H. Vars for provision of bile-cannulated rats and to T. E. Briggs for samples of recrystallized synthetic bile acid conjugates.

TABLE I. Oxidation of Cholesterol-27-C^{14} and Sodium Pyruvate-2-C^{14} by Liver Mitochondria of "Bile-fistula" and Control Rats.

	Animal	Specific activity Ba^{14}CO$_3$ (cpm/mg)* from oxidation of Pyruvate (P)	Cholesterol (C)	C/P
Exp. 1	"Fistula"	6.2	25.9	4.2
	Control	8.4	10.7	1.3
Exp. 2	"Fistula"	2.4	10.2	4.2
	Control	2.1	7.3	3.6

* Corrected for equivalent amounts of mitochondria (mg N).

Exp. 1—Bile was drained 4 days following operation before animal was sacrificed.

Exp. 2—Bile was recirculated 17 days following operation, then drained 3 days before sacrifice.

and final passage through an alumina column. Sodium pyruvate-2-C^{14} was obtained from Atomic Energy Research Establishment, Amersham, England. 3α, 7α, 12α-Trihydroxycoprostane-27-C^{14} was synthesized from triformylchloride and di-isopropyl-1,3-C^{14}-cadmium(12,13). Glycocholic and taurocholic acids were synthesized from cholic acid by method of Norman(14) and recrystallized to constant melting point. These synthetic conjugates contained less than 5% cholic acid as determined by chromatographic separation on paper and paper ionopheresis(15). Bile cannulae, which allowed recirculation of bile into the duct, were inserted in male Wistar rats weighing 150-180 g. The bile was allowed to drain into balloons (a) directly after operation or (b) after recirculation for several days before drainage. Animals were fed *ad lib.* and given 0.5% saline as drinking fluid, and after at least 3 days drainage of bile, were sacrificed and their livers removed and weighed. Livers were taken only from those animals which had given a consistent bile flow through the cannula, for at least 3 days. Washed liver mitochondria and a boiled supernatant fraction were isolated from liver homogenates prepared in 10% (weight/volume) aqueous sucrose and incubated at pH 8.5 with cholesterol-27-C^{14} or sodium pyruvate-2-C^{14}, as previously described(5,9). The carbon dioxide-C^{14} evolved was trapped in 2 N sodium hydroxide, precipitated with barium chloride and counted in liquid scintillation counter as suspension of barium carbonate in a Thixin gel. Nitrogen determinations were carried out by micro-Kjeldahl method and the ammonia determined by direct Nesslerization and by titration.

Results. Table I compares oxidation of cholesterol and of sodium pyruvate by fortified preparations of washed liver mitochondria, obtained from livers of controls and from "fistula" rats which had been drained of bile through open cannulae. There was enhanced oxidation of cholesterol, relative to pyruvate oxidation, by liver mitochondrial preparations from bile-fistula rats, compared with oxidation of cholesterol by mitochondrial preparations from control animals. This is indicated by the ratio C/P, computed as ratio of specific radioactivities of CO_2 (as barium carbonate) isolated from incubations with cholesterol-27-C^{14} and sodium pyruvate-2-C^{14} respectively. This ratio was significantly greater for oxidation by liver mitochondrial preparations from bile-fistula animals than for oxidation by similar preparations from control animals.

When taurocholic and glycocholic acids were added to incubations with mitochondria prepared from normal rat livers, a selective inhibition of cholesterol oxidation was observed. Oxidation of sodium pyruvate and of 3α, 7α, 12α-trihydroxycoprostane (THC) was depressed to a much lesser extent (Table II). Unconjugated bile acids (cholic, deoxycholic, lithocholic acids) and tauro- and glycolithocholic acids inhibited oxidation of pyruvate as well as cholesterol oxidation.

TABLE II. Effect of Added Bile Acids upon Oxidation of Sodium Pyruvate-2-C^{14}, Cholesterol-27-C^{14} and 3α, 7α, 12α-trihydroxycoprostane-27-C^{14} (THC) by Rat Liver Mitochondria.

	Addition	% inhibition observed in oxidation of Pyruvate	Cholesterol	THC
		%		
Exp. 1	Cholic acid	44.5	67.7	
	Taurocholic acid	3.0	60.6	
	Glycocholic "	0.0	47.8	
Exp. 2	Cholic acid	63.5	81.5	65.7
	Taurocholic acid	18.8	45.5	27.0
	Glycocholic "	26.5	49.7	28.0

5 mg bile acids, neutralized with Tris, added to final incubation vol of 12.5 ml in 0.1 M Tris hydrochloride buffer, pH 8.5.

Discussion. Cholesterol is oxidized to hydroxycholanic acids by liver mitochondria *in vitro* with loss of terminal methyl groups of the side chain(5). Thus, formation of CO_2 from these carbon atoms serves as index of cholesterol oxidation and bile acid formation.

These experiments indicate that cholic acid conjugates, but not the unconjugated acid, regulate oxidation of cholesterol *in vitro* as well as *in vivo*(6-8). Oxidation of cholesterol *in vitro* was significantly increased when liver tissue was depleted of endogenous bile salts by drainage of bile and interruption of the enterohepatic circulation. Conversely, when bile acid conjugates were added back to the oxidative system, cholesterol oxidation was decreased. That these were specific effects upon cholesterol oxidation and not just activation or inhibition of all mitochondrial oxidases, was indicated by comparative studies upon oxidation of sodium pyruvate by the same mitochondrial preparations under identical conditions.

These studies of cholesterol oxidation in a cell-free system *in vitro* confirm the *in vivo* findings of a feedback regulation of the transformation of cholesterol to bile acids. The mechanism of this regulatory control cannot therefore be ascribed to alterations in permeability of liver cells to circulating cholesterol, under the influence of conjugated bile salts in portal blood.

3*a*, 7*a*, 12*a*-Trihydroxycoprostane (THC), which has the side chain structure of cholesterol and nuclear structure of cholic acid, is readily oxidized by the rat liver to cholic acid and CO_2(12,16) and has been implicated as intermediate in metabolic conversion of cholesterol to cholic acid. In these experiments, bile acid conjugates hardly depressed oxidation of THC, although oxidation of cholesterol was much inhibited. Evidently, the conjugates do not control cholesterol oxidation by competitively inhibiting enzymes which would metabolize any trihydroxycoprostanes, formed as intermediates in transformation of cholesterol to the bile acids. This would appear to support Bergstrom's hypothesis(17) that the rate-determining step in cholesterol catabolism and bile acid formation is associated with nuclear hydroxylation, especially 7*a*-hydroxylation; and it is this step which is sensitive to the conjugated bile acids in the enterohepatic circulation.

Summary. A feedback mechanism, whereby conjugated bile acids control oxidation of cholesterol in liver, has been discerned *in vitro.* Addition of cholic acid conjugates depressed oxidation of cholesterol, but not that of sodium pyruvate or 3*a*, 7*a*, 12*a*-trihydrocoprostane, by fortified rat liver mitochondrial preparations. Preparations from livers of rats, deprived of bile salts by interruption of enterohepatic circulation, oxidized cholesterol to a greater extent than controls. The site of action of the feedback effect is discussed briefly.

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Effect of Intragastric Temperature Changes upon Gastric Blood Flow.* (24972)

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Local gastric hypothermia caused profound reduction in gastric secretion and peptic digestion(1). The results obtained in its use for management of massive gastric hemorrhage in duodenal ulcer have been reassuring (2). However, the rapid cessation of bleeding observed in these patients suggested that limitation of blood flow, as well as suppression of acid-peptic activity, was a likely participating factor in the results observed. These observations indicated the need for determining the influence of local gastric hypothermia upon blood flow in the gastric wall. No information on this subject has come to our notice. Blood flow studies by dye and radioactive tracer technics were difficult of interpretation in assessing the influence of temperature upon blood flow in an organ with so complex a source of blood supply as that of the stomach. In consequence, a simple direct method similar to that used in measurement of renal and coronary sinus blood flow was employed by which it was possible to note and measure changes in gastric flow at normothermic, hypothermic and hyperthermic temperatures.

Method. Adult mongrel dogs weighing 10 to 20 kg were operated upon under intravenous nembutal anesthesia. Fig. 1 diagrammatically illustrates the manner in which gastric venous return was virtually localized to the splenic vein. Splenectomy was performed so as to maintain the integrity of the gastric venous arcades in the hilum of the spleen. The right gastroepipolic vein was ligated near the pylorus and the splenic vein tributaries, draining the pancreas, were interrupted. In this fashion gastric venous return, with the exception of a small loss to coronary and right gastric veins, flowed through the splenic vein to the portal system. In a second group of animals all gastric veins except those tributary to the splenic were ligated, thus obliging total gastric venous return to pass through the splenic vein. Venous outflow is easily determined and is interpreted as a direct reflection of arterial inflow. The splenic vein was isolated near its confluence with the portal vein; it was divided and the arms of a "Y" shaped cannula were inserted into the ends (Fig. 1). By obstructing the proximal arm of the "Y", the entire venous flow returning through splenic vein was collected. All animals were heparinized (3 mg/kg sodium heparin) just prior to insertion of the cannula. Following completion of the preparation, a thermistor in a No. 23 hypodermic needle was imbedded in the posterior wall of the gastric corpus. The animal was allowed to stabilize for 30 minutes before experiment was commenced. Blood flow was measured during 2-minute periods; first with the stomach empty, subsequently following placement and inflation of a balloon with water at 38°C., and finally during circulation of hypothermic or hyperthermic solution through the balloon. Volumes of warm, cold and hot balloons were the same. The

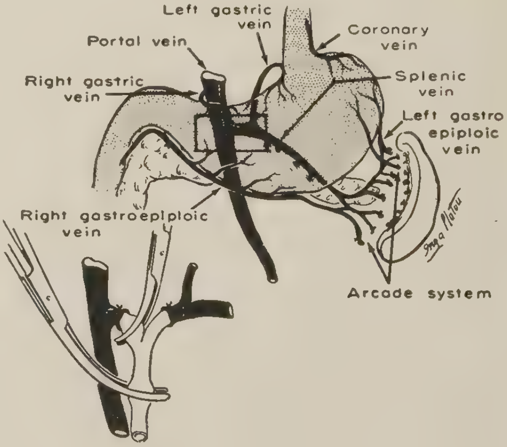

FIG. 1. Preparation for measurement of gastric venous return through the splenic vein.

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TABLE I. Effect of Local Hypothermia on Gastric Blood Flow in 6 Dogs. (Incomplete venous isolation.) Flow—ml/min./kg of body wt.

	Stomach empty	Intragastric balloon inflated		
	S.W.* temp. 38°C	38°C	15°C	Postcooling 38°C
	2.6	2.3	.7	2.4
	2.8	2.5	.6	2.5
	2.9	2.3	1.0	2.7
	3.5	3.1	1.2	2.0
	3.2	3.0	1.1	2.9
	2.7	2.6	.7	2.4
Mean	2.9	2.6	.88	2.5
Range	2.6–3.5	2.3–3.1	.6–1.2	2.0–2.9

* S.W.—Stomach wall.

volume of collected blood was measured and blood was immediately returned to animal through a needle inserted in peripheral vein. Despite marked changes in local gastric temperature, the systemic temperature was maintained at or very near 38°C by external warming or cooling of the animals.

The *results* of experiments upon animals with incomplete gastric venous isolation are reported in Table I. Comparison of mean flows obtained under various conditions described, shows that inflation of the balloon with water at 38°C is associated with slight reduction in blood flow. When intragastric temperature was reduced to 15°C, a mean fall of 66.2% in venous outflow occurred over the 2 minute observation. Rewarming the balloon to 38°C was followed within one hour by return of flows essentially equal to precooling levels. Gradual local warming of stomach wall to 48°C resulted in increased blood flow in all but one dog (Table II). The mean increase at 48°C was 29%. Return of gastric temperature to 38°C produced a concomitant fall in blood flow.

TABLE II. Effect of Local Hyperthermia on Gastric Blood Flow in 6 Dogs. (Incomplete venous isolation.) Flow—ml/min./kg of body wt; intragastric balloon inflated.

	Temp. of stomach wall		
	38°C	43°C	48°C
	2.6	3.5	4.2
	2.6	4.4	4.4
	1.9	2.7	3.4
	3.1	3.3	3.1
	3.8	4.2	4.1
	4.4	4.5	4.8
Mean	3.1	3.7	4.0
Range	1.9–4.4	2.7–4.5	3.1–4.8

The effect of hypothermia on total gastric blood flow is shown in Table III. Mean flow, 5.9 ml/kg/minute, obtained at 38°C is about 40% higher than mean value obtained at similar temperatures in which the venous return was incompletely isolated (Tables I and II). However, depression of gastric blood flow at 15°C is essentially the same (68%) as that in Table I (66.2%). Systemic blood pressures of animals varied only slightly during experiment.

Discussion. A method has been described for estimating changes in gastric blood flow by directly measuring venous flow in the splenic vein. Although an incomplete venous return from the stomach was obtained in animals in Table I, comparison of these data with those of Table III, in which group venous outflow was complete, shows per cent reduction in flow during hypothermia to be essentially the same. The pressure within the splenic vein, taken following splenectomy, was slightly elevated, 3 to 5 cm H_2O pressure, after its cannulation. The elevated splenic vein pressure was probably related to slightly smaller lumen of the "Y" connector, as compared to the vein. Another factor helping to maintain near normal pressures within the splenic vein was the compensatory loss of splenic venous return following splenectomy. The gastric blood flow at normothermic temperatures varied somewhat from animal to animal, particularly in those in which total venous outflow was measured. The wide nor-

TABLE III. Effect of Local Hypothermia on Gastric Blood Flow. (Complete venous isolation.) Flow—ml/min./kg of body wt.

	Stomach empty	Intragastric balloon inflated	
	S.W.* Temp. 38°C	S.W. 38°C	S.W. 15°C
	5.0	5.1	1.6
	4.1	4.0	.8
	8.4	8.6	2.7
	5.2	5.0	1.6
	6.2	5.9	1.8
	7.0	6.5	2.8
Mean	5.9	5.8	1.9
Range	4.1–8.4	4.0–8.6	.8–2.8

* S.W.—Stomach wall.

mal variation in size of stomachs may account for this. Inflation of the intragastric balloon with a solution at 38°C produced only a slight decrease in blood flow. However, initiation of local gastric hypothermia with depression of temperature of stomach wall to 15°-16°C resulted in marked reduction of venous outflow. The stomach wall at these temperatures appeared blanched and slightly cyanotic. This occurrence confirms the observation that decrease in blood flow is due to cooling *per se* and not to pressure effect derived from intragastric distention.

Summary. A method has been described for estimating changes in gastric blood flow by direct measurement of gastric venous return through the splenic vein. Blood flow, attending depression of gastric wall temperature to 15-16°C was decreased by a mean value of 66.2% in those animals where some venous collateral was retained and by 68% where total venous isolation was achieved. Flows increased to 48°C. Return of gastric wall temperature to 38°C was followed by restitution of flows to approximately control levels.

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Failure of Liver to Inactivate Heparin.* (24973)

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Factors involved in terminating the action of intravenously administered heparin have not been established. The brief duration of anticoagulant action of exogenously administered heparin cannot be accounted for on the basis of excretion of the drug in urine(1). Jaques(2) described a heparin inactivating enzyme (heparinase) which he extracted from liver, but other workers(3) have presented evidence indicating that this is not a true enzyme. This report is concerned with *in vitro* and *in vivo* evidence indicating that liver is not selectively involved in terminating the action of exogenously administered heparin.

Methods. Three types of experiments were conducted using either intact mongrel dogs or dog and rabbit liver preparations. The first of these experiments was of the extirpation type in which 3 dogs were anesthetized with sodium pentobarbital (30 mg/kg), their abdomens opened, and 0.5 mg/kg of heparin injected in a saphenous vein. The anticoagulant effect was determined by Lee-White(4) 3 tube method on jugular vein blood samples. When clotting time had returned to normal, the hepatic artery, common bile duct and portal vein were ligated. The hepatic vein was clamped at its junction with the inferior vena cava and approximately 90% of liver was excised. A second dose of heparin (0.5 mg/kg) was then injected in a saphenous vein and the intensity and duration of anticoagulant action determined. The second type of experiment involved administering heparin by continuous infusion into either a branch of portal vein or a superficial saphenous vein in dogs anesthetized with sodium pentobarbital (30 mg/kg). Duration and rate of disappearance of the anticoagulant action of the drug was determined. The third type of experiment involved preparation of an aqueous extract from approximately 500 g of dog liver and from approximately 500 g of rabbit liver according to method of Cho and Jaques(5). The influence of this extract on the metachromatic action of heparin was studied *in vitro,* also according to the method of above investigators. Heparin used was from Lot. No. 4288A assaying 145.7

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TABLE I. Anticoagulant Effect of Sodium Heparin (0.5 mg/kg) in Control and Partially Hepatectomized Dogs.

	Anticoagulant effect (% of control clotting time)					
	30 min. after inj.		60 min. after inj.		90 min. after inj.	
	Control	Hepatectomized	Control	Hepatectomized	Control	Hepatectomized
	410	380	240	300	110	150
	480	380	320	270	150	120
	400	450	280	240	130	130
Avg	430	400	280	270	130	130

Clotting time determined by Lee-White 3-tube method and recorded to nearest 10%. Each dog served as its own control and clotting time before drug administration was from 12 to 18 min.

U.S.P. units/mg, Eli Lilly and Co.† All doses are reported by weight. Solutions were made in physiological saline from the powder just prior to use.

Results. Table I consists of results obtained following injection of sodium heparin in the external jugular vein in control and subsequently partially hepatectomized animals. The Table shows that hepatectomy did not alter maximum anticoagulant effect or duration of anticoagulant effect resulting from administration of a standard dose of 0.5 mg/kg of sodium heparin.

Fig. 1 shows anticoagulant effect of repeated injections into the saphenous vein or of infusion of heparin into the saphenous or

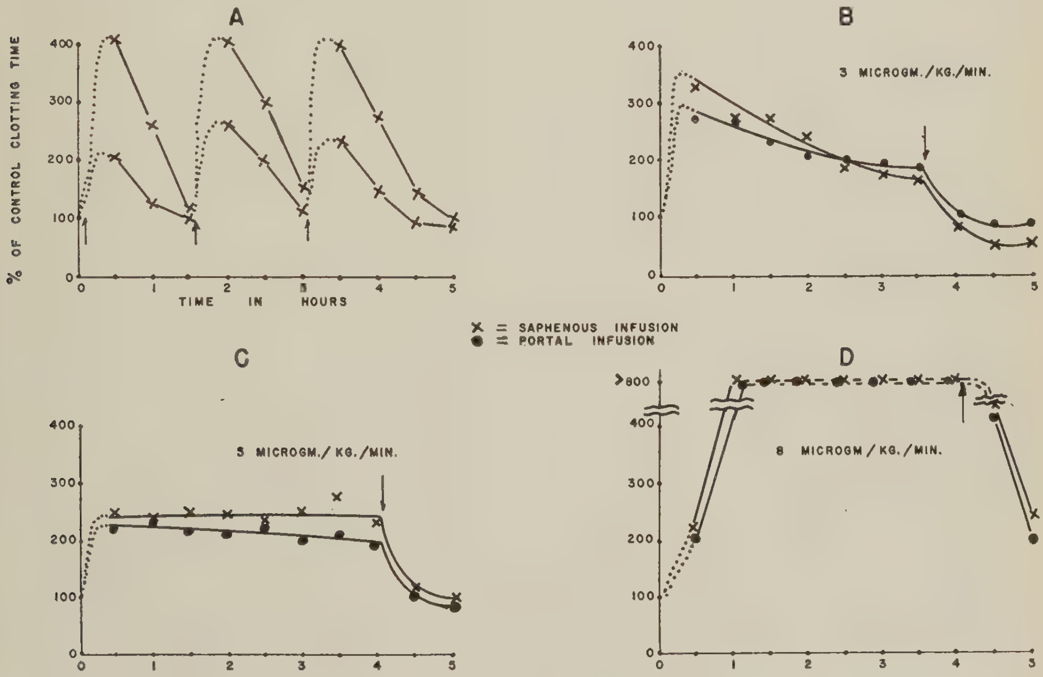

FIG. 1. In Graph A, repeated inj. of 0.25 mg/kg (lower curve) or 0.50 mg/kg (upper curve) sodium heparin are indicated by arrows. In Graphs B, C, and D, infusion of sodium heparin was started 15 min. after priming dose of 0.25 mg/kg of sodium heparin. Infusion rate in all experiments was 0.7 ± 0.05 ml of the solution of sodium heparin in physiological saline/min. as delivered by Merck constant infusion pump. Arrows in Graphs B, C, and D indicate time when infusion solution was stopped. Each curve in Graphs A, B, and C represents mean effect on 4 dogs. Each curve in Graph D represents mean effect on 3 dogs.

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† Heparin was generously supplied by Eli Lilly and Co., Indianapolis.

portal vein in 30 intact anesthetized dogs. Fig. 1, Graph A, shows response to 3 separate injections of 0.25 mg/kg and 0.5 mg/kg of sodium heparin. Intensity of response was greater to the larger dose but duration of anticoagulant effect was not different between the 2 doses. Fig. 1, Graphs B, C, and D, show the effect of infusion of sodium heparin at rates of 3, 5, and 8 μg/kg/minute following a priming dose of 0.25 mg/kg *via* portal and saphenous vein routes. Anticoagulant effect was determined on jugular venous blood samples. Figure 1 demonstrates that rate of metabolism of heparin is approximately 5 μg/kg/minute as far as anticoagulant effect is concerned when the anticoagulant effect is between 2 and 3 times normal. Furthermore, the anticoagulant effect following portal infusion was not different from the effect following saphenous infusion. A deviation in infusion rate of –2 or +3 μg/kg/minute from the 5 μg/kg/minute rate respectively produces a decrease and an increase in anticoagulant action. The curves also indicate that clotting times returned to normal within 30 to 45 mintues after infusion was discontinued.

The *in vitro* effect on metachromatic action of heparin as produced by liver extracts prepared according to method of Cho and Jaques (5) was confirmed. Although these workers had indicated that dog liver does not contain an extractable active material, both dog and rabbit liver used in present experiments showed an effect of decreasing metachromatic activity of heparin. The procedures described by Cho and Jaques were followed both for preparation of liver extracts and for testing of liver extracts on heparin metachromasia. The parallelism between metachromatic activity and antithrombin activity of heparin as described by those authors was also confirmed.

Although original liver extracts (2 preparations each from dog and rabbit) contained some metachromatic activity, control tests showed that it was not significantly altered by the procedure used for testing effect of liver extracts on heparin metachromasia. When liver extracts were incubated at 37° with heparin at pH of 4.0 or 6.0 (MacIlvaine's buffer) and aliquots were removed at 0, 15, and 30 minutes, there was a respective loss of 0 to 10%, 25 to 50%, and 60 to 80%, of metachromatic activity due to heparin. These results are qualitatively similar to those reported by Cho and Jaques although the present liver extracts showed quantitatively greater activity than did preparations prepared by those investigators. However, when pH was raised to 9.0 by addition of sodium hydroxide after incubation and before tests for metachromatic activity were performed, from 80 to 100% of initial metachromatic activity was recovered. These results are similar to those reported by Schuytema and Cushing(3) and indicate that the action of liver extracts on heparin is due to a binding phenomenon and that active heparin can be eluted by appropriate pH adjustment.

Discussion. The strong electro-negative property of heparins is presumed important for their anticoagulant action. All heparin-like substances studied are similarly electronegative and are usually sulfated or sulfonated compounds. Binding of heparin with cationic dyes such as toluidine blue and azure A or with protamine abolishes the anticoagulant action of the drug. Action of drug both as anticoagulant and antilipemic agent is believed to involve binding or reaction of heparin with plasma or tissue co-factors resulting in active antithrombin, antithromboplastin, and antilipemic complexes. Eiber and Danischevsky(6), using heparin tagged with S^{35}, obtained evidence which indicated that short duration of action of heparin is neither the result of excretion of the drug from body nor of selective deposition in any specific type of tissue which they tested (liver, lung, spleen, kidney). It therefore seems possible that the action of heparins is terminated by non-selective deposition in inactive and probably bound forms following which the drug is only slowly excreted in the urine in inactive forms.

Cho and Jaques(5) recognized that heparin may be absorbed on protein in tissue extracts and thus removed non-enzymatically. The present experiments demonstrate that our liver extracts by the method of those investigators are active in binding heparin. Furthermore, *in vivo* experiments reported here indicate lack of a highly active mechanism in liver for terminating heparin anticoagulant action in the dog. It is possible that surgical procedure altered susceptibility of animal to the anticoagulant effect of heparin. This possibility has not been adequately tested, because currently available chemical methods for determining blood heparin concentrations at levels we used have not been found sensitive enough.

Summary. Evidence obtained by 2 methods in the *intact* dog indicate that liver is not involved in terminating the anticoagulant action of exogenously administered heparin. Liver extracts reported to contain a heparin inactivating enzyme were found to influence heparin only by a binding mechanism.

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Absorption of Heparin from the Intestine.* (24974)

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Oral administration of heparin does not result in systemic anticoagulant effects of this drug, and anticoagulant activity can be recovered in the feces(1). Although there is some evidence(2) that sublingual wafers of sodium heparin will result in a systemic anticoagulant effect this work has not been confirmed. Fuller(3) has indicated that potassium heparin administered sublingually will decrease intensity of post alimentary hyperlipemia without producing an effect on clotting time; however, in similar studies Engelberg(4) found no significant sublingual absorption in 18 of 21 patients. This report describes experiments which show that small amounts of heparin† can be absorbed from intestinal lumen of the dog and that this is dependent at least in part on the pH of heparin solution.

Methods. Sixteen adult mongrel dogs, 10 to 15 kg were used. Each animal was anesthetized with sodium pentobarbital (25 mg/kg intravenously). The abdomen was opened in midline, and 10 to 12 inch segment of duodenum located about 12 inches distal to pylorus was clamped, ligated and sectioned on both ends, leaving mesenteric blood supply intact. The duodenal loop was irrigated with approximately 200 ml of physiological saline. Test buffer solutions or buffer plus sodium heparin solutions were instilled into the loop. The loop was then replaced in the abdomen, and abdominal incision was closed with clamps. Systemic anticoagulant effect was determined by the Lee-White(5) 3-tube method on femoral or external jugular venous blood before and at ½ hour intervals following instillation of solutions into the intestinal loop. Analyses for residual heparin in the loop were performed according to the phenol extraction method of Monkhouse and Jaques(6) as applied to metachromatic assay (7). Undiluted MacIlvaine's (citric acid-disodium phosphate) buffer(8) was used for buffer solutions. Heparin sodium, Lot No. 4288A, potency - 145. 7 U.S.P. units/mg, Eli Lilly and Co.† was used and solutions prepared from this powder just before use.

Results. Sodium heparin in doses of 100 or 200 mg in 20 ml of physiological saline (pH 5.2) instilled into intestinal loop in 4 dogs did not result in a significant increase in coagulation time during the following 8 hours.

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† Heparin was generously supplied by Eli Lilly and Co., Indianapolis.

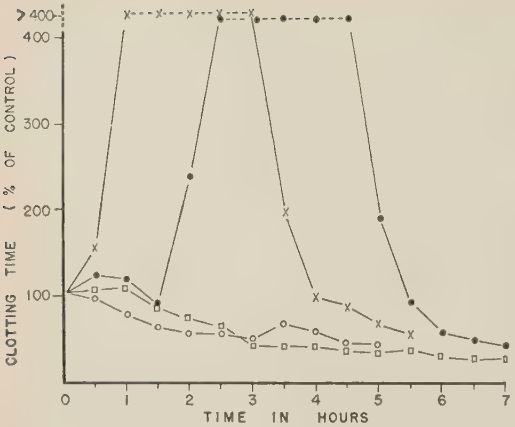

FIG. 1. Influence of acid buffer and saline on absorption of heparin from duodenal loop of dogs. Each curve represents a single separate dog. ● = 200 mg heparin in 20 ml saline instilled at 0 time followed at 1½ hr by instillation of 20 ml buffer, pH 4. × = 200 mg heparin in 20 ml buffer, pH 4, instilled at 0 time. ○ = 20 ml of buffer, pH 4, instilled at 0 time, at 3 hr loop washed with 200 ml saline, at 3¼ hr 200 mg heparin in 20 ml saline instilled. □ = 200 mg heparin in 20 ml saline instilled at 0 time. Control clotting times in all dogs were between 12 and 16 min.

Rather, coagulation time gradually became shorter during this time.

Fig. 1 and Table I show the effect of altering pH of diluent on absorption of a standard dose of 200 mg of sodium heparin. In presence of acid buffer of pH 4.0, a significant systemic anticoagulant effect was obtained. Instillation of buffer only, 2 to 3 hours after heparin in physiological saline had been instilled in the intestine, also resulted in significant systemic anticoagulant effect. Instillation and then removal of only the acid buffer followed by instillation of heparin in physiological saline did not result in a systemic anticoagulant effect. Instillation into the intestinal loop of heparin in alkaline buffer (pH 8.0) did not result in a systemic anticoagulant effect.

pH and concentration of heparin in luminal contents at end of experiments in which acid buffer was used was determined. Four to 5 hours after heparin in acid buffer (resulting solution pH 4.0) had been instilled, the luminal contents had a pH of 6.0 to 6.5 and 40 to 80% of initially instilled heparin was recovered from the luminal contents as determined by extraction and metachromatic activity. Four to 7 hours after heparin in physiological saline (resulting solution pH 5.2) had been instilled into the intestinal loop, luminal contents showed a pH of 6.0 to 6.5 and 80 to 100% of the initially instilled heparin was recovered.

Gross inspection of intestinal loops at end of experiments showed no evidence of general mucosal inflammation. However, there was a mild local inflammatory response adjacent to area which was traumatized by application of ligatures at each end of loop.

Discussion. Schanker *et al.*,(9,10) and Hogben *et al.*,(11) studied the influence of pKa on absorption of drugs from stomach and intestine of rats and from stomach of humans. Their results indicate that absorption of the several drugs studied was dependent on degree of un-ionization; that is, agents in the ionized state were not absorbed, whereas those in the un-ionized state were absorbed from gastric and intestinal lumen. Most drugs studied were of relatively small molecular weight as compared with heparin. However, they also had evidence which indicated that molecular size may not be an important factor. In general, most drugs studied were readily absorbed except for stronger acids (pKa = <2.9) and stronger bases (pKa = >8.4)

TABLE I. Influence of Buffer at pH 4.0 and Saline on Absorption of Heparin from Duodenal Loop of Dogs. Clotting time of venous blood was recorded to nearest 10% of control. Each dog acted as its own control. Control clotting times were between 12 and 18 min.

	Hr after instillation						
	.5	1.0	1.5	2 to 3	4.0	5.0	60
	Clotting time of blood as % of control						
	After 200 mg sodium heparin in 20 ml buffer, pH 4						
	120	>400	>400	>400	100	80	50
	100	280	320	”	200	90	
	240	>400	>400	”	100		
	200	350	”	”	250	70	40
	120	>400	”	”	110	90	
Avg	160	”	”	”	150	80	50
	After 200 mg sodium heparin in 20 ml saline						
	100	120	100	60–80	50		
	110	120	80	50–80	70		
	90	90	70	”	30	20	
	120	80	80	”	80	50	40
Avg	110	100	80	”	60	40	40

which were absorbed only slowly.

Charles and Todd(12) estimated that the barium heparin contained 5 sulfuric acid residues and 2 carboxyl groups/molecule. They believed that the carboxyl groups were unsubstituted. In the current experiments it is probable that only the carboxyl groups are influenced by lowering the pH to 4.0. The state of ionization of the carboxyl groups therefore, may be significant with respect to absorption of heparin.

Our experiments indicate that there is no significant destruction of heparin in the intestinal loop. Approximately 100% of the heparin initially instilled in the gut can be accounted for by estimating amount absorbed. This estimation is based on a metabolic rate of 0.5 μg/kg/minute during the interval that anticoagulant activity was present and measurement of the free heparin remaining in the gut after a 5 to 7 hour experiment. These results indicate that during the experiment when the initial pH in the lumen of intestine was about 4.0, absorption of approximately 10% of instilled heparin took place. During the experiment, however, pH of the luminal content gradually returned toward neutrality, and absorption of drug then ceased.

Summary and conclusion. Sodium heparin is partially absorbed from the lumen of a washed duodenal loop in the dog following addition of acid buffer of pH 4.

The technical assistance of Carolyn S. Spaeth is gratefully acknowledged.

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Fat Deposition in Musculature of the Genetically Dystrophic Chicken.*
(24975)

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Asmundson and Julian(1) reported that an inherited muscular dystrophy prevented New Hampshire chickens from raising their wings and rising from a flat surface when laid on their backs. The affected birds were homozygous for a recessive autosomal gene (am), and had wider breasts than is normal in this breed. Our exploratory biochemical determinations suggested that this condition in the chicken is similar to certain forms of muscular dystrophy in man(2) characterized histologically by disarrangement of musculature and by large numbers of fat cells. In biochemical characterization of this condition in the chicken, fat deposition was the first major area of interest. When percentage of crude fat in the wet muscle sample was determined, excess fat deposition was associated with physical manifestations of muscular dystrophy, and was a function of age. Further, a sexual dimorphism was discovered, with females generally de-

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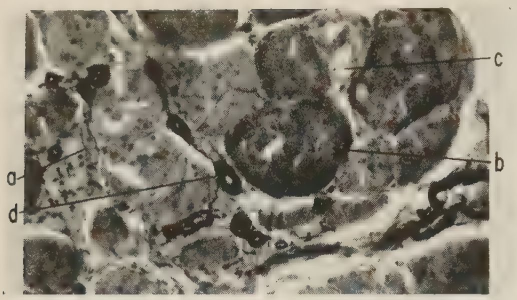

FIG. 1. Superficial pectoral muscle of genetically dystrophic male N.H. chicken, 22 mo of age, fat stain, ×100, containing 27% fat.

positing more fat than males.

Methods. Chicks were brooded and grown to maturity in wire batteries, then placed in laying cages and fed standard ration. Muscle samples to be analyzed were thoroughly minced with scissors, weighed into dry beakers, and dried 18-20 hours in vacuum oven at 100°C and 5 inches of mercury pressure. The dried samples were submerged in petroleum ether (60°-110° B.P.) or toluene and allowed to stand for about one hour. The supernatant was removed and the process was repeated twice, for periods of 3-4 hours, and finally overnight. If necessary, the process was continued (using 2-4 hour periods) until supernatant was clear. The samples were then dried again, by being left overnight under 250-watt heat lamp, followed by 2 hours in oven at 100°C. Fat content is expressed as per cent of wet sample. When muscle size permitted, duplicate samples were assayed. This technic, when compared with one using a fat extraction apparatus (Soxhlet), was equally reproducible (± 10%). To compare histology with fat content, the tissues were fixed in slides in 10% formalin and stained with Oil Red OB stain (Fig. 1). Muscle samples were of 2 origins: 1) from randomly selected birds of the dystrophic strain (NH3A) and of a normal strain of New Hampshires (NH2); and 2) from the anterior superficial pectoral muscle, obtained by serial biopsies at intervals, from 15th through 34th week, from a population consisting initially of 12 NH3A ♂, 9 NH3A ♀, 1 normal ♂, and 2 normal ♀. In several instances, per cent of fat was determined for superficial pectoral, deep pectoral, biceps femoris, and sartorius muscles. In the biopsy series, only the superficial pectoral muscle tissue was analyzed.

Results. The fat content of the superficial pectoral muscle is shown in Table I. Fat content could be considered abnormal only when it exceeded 3%. The deep pectoral muscle appears to mirror the superficial pectoral muscle in fat content (Table II). In normal New Hampshire chicken, fat content of the sartorius and biceps femoris is at least equal to that of pectoral muscles. In the dystrophic bird, the fat content of these 2 muscles does not increase; indeed, in some cases it appears to decrease.

In the superficial pectoral muscle, fat has been seen in 3 forms. These forms are indicated in Fig. 1 as follows: (a) Interfascicular fat is in the form of fat cells that may possibly replace muscle fibers. This has been described in a number of natural and experimental muscular dystrophies of man(2). (b) Small fat droplets can be seen distributed throughout the sarcoplasm of some muscle fibers. The number of droplets varies between fibers; as a result, coloration of individual fibers of a section stained specifically for fat varies markedly. (c) Large intrafibrillar fat vacuoles, possibly smaller droplets coalesced, are situated within the muscle fibers. These are responsible for some vacuolated spaces in routine microscopic preparations. (d) The extremely dark-stained areas are merely air-bubble artifacts.

To date, it has been impossible to demonstrate fat droplets in superficial pectoral muscles of normal New Hampshire chickens. This failure is consistent with the very low fat levels reported above for such muscles.

Discussion. The crude fat content of the superficial pectoral muscle was not significantly different between normal New Hampshires (NH2) and the dystrophic strain of the same breed (NH3A) until sexual maturity (15 to 19 weeks of age). When sex differences were considered in each strain, fat deposition proved significantly different between any 2 groups at the 5% level beginning at 15 weeks old except in normal birds, in which sex differences were significant only at 34 weeks of age. At that age, dystrophic males were significantly different from normal birds only at

TABLE I. % Fat of Wet Sample Weight in Superficial Pectoral Muscle.

	NH3A ♂			Normal ♂			NH3A ♀			Normal ♀		
Age (wk)	No. samples	% fat	E*	No. samples	% fat	E	No. samples	% fat	E	No. samples	% fat	E
4	10	1.7	.2	10	2.5	.3	10	1.9	.2	10	1.1	.3
8	10	2.4	.2				24	1.4	.1			
15†	19	1.4	.2	1	.01	.0	30	2.5	.4	2	.2	.2
19	12	3.6	.7	1	.2	.0	9	8.3	2.1	2	.2	.1
24	11	4.0	.6	1	1.1	.0	11	13.7	2.3	1	.9	.0
34	9	5.1	2.0	1	1.3	.0	10	18.0	4.7	2	2.1	.1
52+	2	6.3	.1				8	25.8	6.4			
78	2	20.6	.5	14	1.4	.4						
84	1	21.0	.0				4	46.6	7.2			

* Stand. error of mean.

† Earliest age at which all results are significantly different from each other (at 5% level) except between Normal ♂ and Normal ♀.

the 10% level, but this can be attributed to a single bird that varied markedly from all other dystrophic males. Fat deposition in dystrophic muscles increased with age.

The data show sexual dimorphism of fat deposition in the superficial pectoral muscle of the dystrophic chicken. This is not evident in the normal chicken, although general lipid metabolism of the bird is influenced by the ovarian estrogenic hormone(3). The data suggest an interactive effect of the endocrine system upon fat deposition in the pectoral muscle of the dystrophic chicken, and ultimately upon severity of the dystrophy syndrome.

Standard errors of means for the data (Table I) reflect a great variation in fat deposition among the dystrophic birds. The crude fat level in certain NH3 males and females was as low as in normal birds, or even lower. A prolonged hypertrophy of muscle tissue is seen in such cases. This suggests that 2 or more strains may be isolated from the existing dystrophic population, based on age at which fat deposition increases markedly.

TABLE II. % Fat of Wet Sample Weight in Muscle.*

Muscle	NH2 (7♂)	NH3A (3♂)
Sartorius	5.58	.66
Biceps femoris	1.30	.54
Deep pectoral	.74	4.38
Superficial pectoral	1.36	10.08

* Birds were 76-84 wk of age.

The bird's inability to rise from a flat surface when laid on its back preceded the symptom of fat deposition by at least 10 weeks.

Summary. Genetically dystrophic chickens contain significantly more crude fat in the superficial pectoral muscle (% of wet sample) than do normal chickens. An increased fat deposition is noted as early as 15 weeks of age and is associated with physical manifestation of dystrophy resembling certain muscular dystrophies in man. Females deposit more crude fat than males.

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Purification of Human Pituitary Follicle Stimulating (FSH) and Luteinizing (LH) Hormones.* (24976)

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A number of investigators reported partial purification of human gonadotropin and follicle stimulating hormone (FSH)(1,2,3). Steelman *et al.*(3) showed that a simple extraction of acetone dried pituitaries and precipitation with alcohol resulted in a 50-100 fold purification of gonadotropins. Since the initial publication it has been possible to separate FSH and luteinizing hormone (LH) activities and prepare highly active fractions.

Methods and results. FSH was assayed by the method of Steelman and Pohley(4) based upon ovarian weight increase in intact immature female rats treated for 3 days with human chorionic gonadotropin. In all assays samples were dissolved in 0.9% saline containing 0.5% gelatin at pH 6-7. LH was measured in immature hypophysectomized male rat using increase in ventral prostate weight as index of activity. Animals were injected (24 hours after operation) once daily for 4 days and sacrificed on fifth. Total gonadotropin activity was determined by increase in uterine and/or ovarian weights in immature female rats and mice. The assay schedule consisted of 2 subcutaneous injections a day for 3 days and autopsy on fourth day. Chromatography with *carboxymethyl cellulose* (CMC). Sixty mg of human gonadotropin concentrate(3) was dissolved in 60 ml of water and adjusted to pH 6.1 with dilute ammonium hydroxide. This was placed on a column of CMC (0.4-0.5 meq/g) (10 x 200 mm) previously equilibrated at pH 6.1 with 0.01 M ammonium acetate buffer (Fig. 1). The sample was followed with pH 6.1, 0.01 M ammonium acetate. Elution of retained fraction was accomplished with 1 M ammonium acetate and was designated fraction CMC-B and contained LH. The non-retained fraction (CMC-A) had about 80% of protein and all FSH activity. Fraction CMC-B was lyophilized and reworked on CMC under identical conditions, except a gradient (1 M NH_4Ac through 200 ml reservoir of 0.01 M NH_4Ac) elution was employed. This resulted in 2 well separated peaks—CMC-B-1 and CMC-B-2. Fraction CMC-B-1 which emerged first contained practically all the LH activity, while CMC-B-2 had very low activity. *DEAE-Cellulose.* Fraction CMC-A was adjusted to pH 7.0 with dilute ammonium hydroxide and placed on a DEAE-cellulose (0.5 meq/g) column (8 x 150 mm) equilibrated at pH 7.0 with 0.01 M ammonium acetate (Fig. 2).

All FSH activity and most of protein were adsorbed. After washing with 0.01 M buffer, a gradient of 0.75 M NH_4Ac through 200 ml of 0.01 M NH_4Ac was applied. The FSH fraction emerged first and was extremely active (50 x standard). Fraction B was less than 10 times standard. Although optical density at 280 mμ was used to estimate protein content of individual tubes, the protein content of fractions from pools of tubes was determined by a microbiuret method(5) using crystalline bovine albumin as a standard. All activities were expressed in terms of biuret protein. Folin-Lowry determinations(6) were made on selected samples and in general agreed with biuret values, although they tended to be slightly higher.

Attempts to rechromatograph DEAE fraction A resulted in considerable loss in biological activity and no increase in specific activity of any fraction. This confirms observations made with swine FSH(7).

Table I summarizes gonadotropic activities of the various fractions. It was of particular

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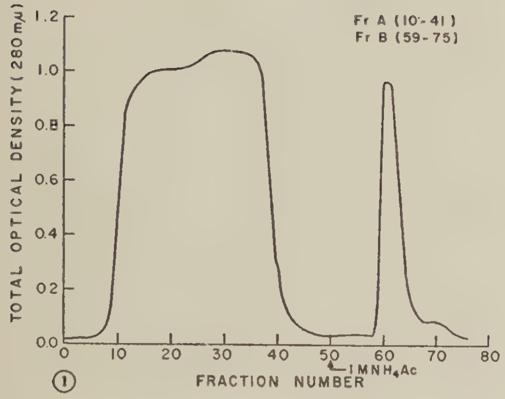

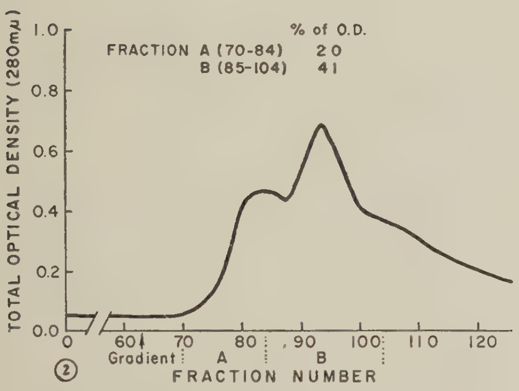

FIG. 1. Chromatography of human gonadotropin on CMC. Fraction volume was 2 ml and total optical density was observed optical density (280 mμ) multiplied by sample volume.

FIG. 2. Chromatography of human FSH on DEAE-cellulose.

interest that human pituitary LH was approximately 10 times as active as pure sheep LH. A total dose of 500 mμg gives at least 100% increase in ventral prostate weight in hypophysectomized male rats. Sufficient human LH has not been available to conduct adequate physicochemical studies; however, the CMC-B-1 did not appear to be homogeneous. Steelman and Segaloff(8) reported that purified equine gonadotropin concentrates have LH activities of 5-7 times the Armour Standard (227-80).

Human pituitary FSH appeared to be at least as potent as swine and sheep FSH(8). On the basis of published data, the FSH preparation reported here is at least 10-20 times as active as those of Li(1) and Gemzell(2). Unfortunately, the same reference standard was not used by the 3 laboratories. Li(1) has proposed the use of a slope unit based on the assay of Steelman and Pohley(4). Unfortunately, this predicates the use of a biological end point rather than a standard reference preparation. The variation in responsiveness of the animals renders this of doubtful value as a quantitative measure of FSH activity. Twelve consecutive FSH assays conducted in the laboratory of one of the authors (SLS) were analyzed and the slope unitage determined for the reference preparation (264-151-X). A range of 200-750 units/mg was found. Fraction DEAE-A possessed 10,000-20,000 slope units per mg. It was estimated that a total dose of 2 μg of DEAE-A will, under normal assay conditions, produce approximately the same response as 50 μ of the Li Fraction F. Table II summarizes a typical FSH assay of 2 separate DEAE-A fractions of human FSH. The slopes of the dose response curves of the standard and unknown do not differ significantly.

It has been possible to prepare human LH which was relatively free of FSH. Sufficient LH has not been available to assay for FSH at very high dosage levels. So far it has not been possible to prepare FSH free of LH. Even recycling the FSH fraction from the CMC column on fresh CMC did not remove additional LH. Either the remaining LH has different properties from the one retained by

TABLE I. Gonadotropic Activities of Human Pituitary Fractions.

Fraction	FSH X Standard*	LH X Standard†	Gonadotropin R.U./mg‡	Gonadotropin M.U./mg§
Starting material	8–10	3–5	200– 400	
CMC–A	10–12	.5–1.0	100– 200	
–B-1	<.5	10	500–1000	300–500
DEAE–A	50	1–1.5	500–1000	300–500

* Armour Standard 264-151-X. † Armour Standard 227-80. ‡ One rat unit = 100% increase in uterine wt. § One mouse unit = 100% increase in uterine wt.

TABLE II. Activities of Highly Purified Human FSH, 5 Animals/Series.

Preparation	Total dose* (μg)	Ovarian wt (mg)†	Potency X standard
Control		39.0 ± 3.9	
Standard (264-151-X)	100	79.6 ± 8.8	
Idem	200	102.6 ± 5.5	
FMA	2	80.1 ± 6.7	54
"	4	110.6 ± 9.9	
FJC	2	75.5 ± 6.5	56
"	4	125.6 ±18.2	

* Each animal received total of 40 I.U. of human chorionic gonadotropin plus indicated amount of FSH.

† ± stand. error of mean.

CMC or, it is inherent in the molecule of FSH. It should be recalled that pregnant mare serum gonadotropin has both FSH and LH activity. Indeed, the preparations reported by Li(1) and Gemzell(2) both contained considerable LH. Ward *et al.*(9) and Li and Squires(10) have reported 2 types of LH from sheep pituitaries. In the case of Ward *et al.*(9) both were adsorbed on CMC under the conditions cited in this paper. In fact, it was proposed that one was derived from the other, and it is possible that they are identical with the α and β LH of Li and Squires(10).

Our important finding was that both human FSH and LH were highly active in causing increases in uterine weights of rats and mice. For many years, the standard method for determining gonadotropin content of human urine has been the increase in uterine weight in immature mice(11). This has erroneously been called FSH. As can be clearly seen in Table I, both FSH and LH were active. Recently(10) it has been shown that human urinary gonadotropins can be separated into FSH and LH fractions, and that both have uterine weight activity. The two hormones had chromatographic behavior similar to their precursors from the pituitary. Thus, it should be possible physically to separate and biologically assay FSH and LH in the urine of humans. This would materially aid the clinician in accurate diagnosis of many gonadal and pituitary dysfunctions.

Summary. FSH and LH from human pituitaries have been isolated with high specific activities. Both FSH and LH had uterine weight increasing activity in immature rats and mice. The FSH had approximately the same activity as porcine and ovine FSH, however, LH was 10 times as active as pure ovine LH.

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Sensitivity of Mice to *Neisseria gonorrhoeae* Lipopolysaccharide After Injection of Ehrlich Ascites Tumor Cells. (24977)

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This report is part of studies(1,2) concerning properties of protein and nucleic acid-free lipopolysaccharide phosphoric acid ester endotoxin (LPSP) obtained from *N. gonorrhoeae.* Investigators have found that lipopolysaccharides of certain gram negative bacteria induce hemorrhage and necrosis in certain types of tumors of animals and man(3). The malignant tumors responded to a single injection of lipopolysaccharide in the above fashion. In this study we were interested to know whether LPSP of *N. gonorrhoeae* would offer protection against or enhancement of the toxic nature of Ehrlich mouse ascites carcinoma cells.

Materials and methods. Ehrlich ascites tumor was maintained in male CF1 mice by transferring 10^6 tumor cells, suspended in 0.1 ml sterile saline, into the peritoneal cavity. The mice were sacrificed by chloroforming 8 or 9 days after inoculation. LPSP was injected intraperitoneally 18 hours after injection of 0.1 ml tumor cell-suspension (10^6 cells). Five different LPSP concentrations, increasing logarithmically, with 5 mice for each concentration were used. Preparation and chemical nature of LPSP-endotoxin has been described(4). The LPSP powder was dissolved in a small volume of 0.05 N sodium hydroxide. The resulting solution was adjusted to pH 7.4 with 0.1 N acetic acid, diluted to desired concentration with sterile distilled water and administered intraperitoneally. Karber's method was employed for calculating LD_{50} values(5). Experiments were carried out as follows: each group of mice was sub-divided into LPSP-control, ascites-control, and ascites-LPSP groups. The LD_{50} value for the LPSP varied between 300-500 μg.

TABLE I. Effect of LPSP on Mouse Ascites Tumor.

Exp.	Range of LPSP (μg)	Mortality (%) in days* 5	10	15
1	300	78	88	100
1 C	"	36	40	40
2	200	70	80	100
2 C	"	12	12	12
3	50–150	50	78	100
3 C	"	4	8	8
4 AsC	none	0	16	32

Exp. 1-3 represent mice which received both ascites tumor cells and LPSP. Exp. 1C-3C represent LPSP controls. Exp. 4 AsC represents mortality rate of mice receiving ascites tumor alone.

Total No. of mice employed were 175.

* Mortality figures represent death rates after No. of days given.

Results are summarized in Table I. Three different LPSP ranges varying from 50-300 μg LPSP have been studied. Within these ranges most mice (50-78%) which received ascites tumor prior to LPSP died within 5 days. Death rate in controls which received LPSP was 4-36% within 5 days. In a typical experiment (not listed in Table I) 18 hours after mice were inoculated with 10^6 ascites tumor cells, the groups received 5 different concentrations of LPSP varying from 10.5-40 μg. These quantities of LPSP did not increase the sensitivity of mice to ascites tumor or influence its effect on mice bearing tumors. Our results definitely indicate that mortality rate of mice infected with Ehrlich ascites tumor cells was increased by sublethal doses of LPSP from *N. gonorrhoeae.* This increase in mortality rate commenced 24 hours after injection of LPSP. Mortality rates of mice which received ascites tumor alone was 32% between 10-15 days after ascites inoculation.

Summary. 1. The mortality rate of mice infected with Ehrlich ascites tumor cells was increased considerably by sublethal doses of LPSP. 2. Very small quantities of LPSP had no effect on such mice.

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Effect of Quinidine on Ventricular Fibrillation in Hypothermic Dogs.* (24978)

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Ventricular fibrillation occurs regularly during profound hypothermia in dogs(1). Gollan(2) has prevented this arrhythmia by a combination of quinidine, slow rewarming period, prevention of left heart distention, and control of rate of coronary perfusion. In experiments reported below we tested the efficacy of quinidine alone as a means of preventing V. F. in hypothermic dogs.

Methods. Hypothermia was induced in healthy, mongrel dogs anesthetized with pentobarbital. Extracorporeal circulation was carried out with a pump-oxygenator-heat exchanger device as described elsewhere(3). A thermistor was placed in the esophagus and flow through the extracorporeal circuit set at 50 ml/min/kg until esophageal temperature fell to 20°C. Flow was then reduced to 25 ml/min/kg. E. K. G., E. E. G. and femoral blood pressure were recorded. The chest was opened only when electrical defibrillation was needed or when other direct procedures on the heart were carried out.

Methods. Eleven dogs (Group I) were cooled to temperatures of 6-10°C without receiving quinidine. Fifteen dogs (Group II) were similarly cooled but received 28-30 mg/kg quinidine: HCl 10-15 minutes before start of cooling period. The quinidine was given i.v. in doses of 100 mg every 5 minutes until QRS interval doubled or until PR interval increased to 40-60 mS.

Result. All 11 dogs in Group I developed ventricular fibrillation either during cooling, rewarming, or both periods. In all dogs, electrical defibrillation was successfully accomplished at temperatures of approximately 30°C. Dog 4, placed on a pacemaker, fibrillated at 16° on rewarming, even though heart continued to respond up to point of fibrillation. In 5 runs where cooling fibrillation did not occur, the heart spontaneously stopped. When it fibrillated on rewarming, there were usually 3 or 4 slow rhythmical beats before ventricular fibrillation began. Acetylcholine (3 mg/kg) Mecholyl (3.5 mg/kg) and TEA (3 mg/kg) were ineffective in preventing onset of ventricular fibrillation in single experiments. Maintenance of left auricular pressure below 15 mm Hg was similarly ineffective in one experiment.

In 15 dogs in Group II, there were only 2 instances (dogs 6 and 7) of fibrillation. One was due to inadequate initial dose of quinidine and second was probably due to too low quinidine blood levels on the fourth hypothermic episode. Complete cardiac standstill occurred at about 8.5°, though in one instance this was at 13°C. On rewarming, cardiac action returned at about 16° varying from 11° to 19°. Following rewarming, there were no EKG abnormalities to suggest residual quinidine toxicity; 11 dogs were allowed to survive.

Discussion. Large doses of quinidine have proven effective in preventing ventricular fibrillation during profound hypothermia of 10°C or lower, when this is accomplished by core cooling with pump-oxygenator-heat exchanger system. Quinidine, to achieve this effect, was administered intravenously until suppressive effect was noted in the EKG as shown by changes in QRS and PR intervals (4). This occurred well before any signifi-

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TABLE I. Prevention of Ventricular Fibrillation with Quinidine during Profound Hypothermia.

Dog	Period of hypothermia	Low temp., °C	Cardiac arrest, min.	I.V. quinidine, mg/kg	V.F.
9	1	9	13	30	0
10	1	6	22	35	0
11	1	6.5	20	28	0
13	1	5	26	32	0
6	1	1	19	20(I.M.)	Rewarm
	2			25	0
7	1	8.8	9	30	0
	2	9	8		0
	3	8.2	7		0
	4	9	9		Rewarm
8	1	8.2	19	30	0
	2	8.8	13		0
	3	9.5	11	10	0
	4	10	10		0
18	1	7	60	30	0
21	1	7	74	30	0
22	1	7	68	30	0
23	1	7	69	30	0
24	1	7	74	30	0
26	1	7	60	30	0
27	1	5.5	65	30	0
28	1	6	68	30	0

V.F.—Ventricular fibrillation.

cant hypotensive effect of drug.

The mode of action of quinidine in prevention of ventricular fibrillation in profound hypothermia is not known. It is possible that suppressive action of quinidine may prevent premature firing of a stimulus or premature response to stimuli when there are still critical temperature gradients in heart that prevent uniform myocardial response(5).

Complete circulatory standstill for as long as one hour is tolerated at temperatures below 10°C. This with demonstration of simple method of preventing ventricular fibrillation suggests many possibilities for surgical applications of this technic.

Summary. In a series of animals with esophageal temperatures reduced to 10°C or lower by core cooling, ventricular fibrillation has been regularly prevented with intravenous infusion of quinidine.

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Exogenous Timing of Rat Spontaneous Activity Periods.*† (24979)

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While numerous investigations have resulted in descriptions of periodic patterns of spontaneous locomotor activity in mammals in constant conditions, particularly those with periods approximately 24-hours in length, very little progress has been achieved in resolving the mechanism by which these mammalian periodisms are timed. Brown, Shriner and Ralph(1), studying a male white rat, both in conditions of constant darkness and constant low illumination, found 24-hour cycles under the former conditions and 25¼-hour cycles under the latter. Utilizing the rat's 25¼-hour overt rhythm of running to randomize the overt running relative to time of solar day (24-hours) and lunar day (24.8-hours), these investigators found, underlying the overt 25¼-rhythm, both solar-day and lunar-day rhythmic components. The more conspicuous was lunar-day one, with rat running about 3 times as much at lunar nadir as at lunar zenith. It was postulated that these natural-period cycles constituted reference

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[†] Authors express appreciation to Phyllis Holter and Nancy Knutsen, who assisted in obtaining the data.

clocks for timing rat's overt running cycles. However, the highly dominant overt 25¼-hour activity pattern contributed such variance to data as to preclude proper quantitative analysis of any existing interrelationships among the various periodisms. The following study was carried out to determine reproducibility of earlier results, and to attempt to learn more concerning this very fundamental problem. In view of the special nature of the results obtained, it should be emphasized that this study does not involve a selected case; it is the first and only study of the rat carried out in our laboratory since the earlier reported one.

Methods. One adult male white rat was placed in a modified Hemmingson-Krarup activity recorder(2) on Jan. 11, 1959. The recorder was kept in photographic darkroom continuously illuminated dimly (ca. 10 lux) through voltage-regulated power supply. Temperature was thermostatically controlled at 24°C. The recorder was screened by black cloth from persons working irregularly, in the same room, and who, on 6 or 7 occasions during the study turned off the lights for 2 minutes to an hour. The running wheel activated, through micro-switch, a signal-magnet operated pen registering in another room, periods of wheel rotation. The rat had always available excess of food and water. Values indicating number of differentiable running periods for each hour of 60 consecutive days (Jan. 12 through Mar. 12, 1959) were obtained.

Results. Tabulation of results revealed a conspicuous overt, lunar-day rhythm of running, the major period of running occurring 50 minutes later each day to yield a clear monthly repetition of daily patterns of activity. Maximum activity occurred over noon hour during each period of full moon. The data for each calendar day were consequently arranged to give their proper relationship as hours of lunar-day, with 12th hour being that of lunar zenith and 24th hour that of lunar nadir. This Table forms Fig. 1. It is evident that rat's spontaneous running is high for whole 60-day period during hours the moon is below the horizon and low when it is above. A gradual drift of 12 minutes/day in either direction away from precise lunar-day frequency would have resulted in period of activity moving completely from lunar "night" to lunar "day" over the 60-day period. Not only did such a phase drift not occur, but there is no suggestion of even such a slight phase drift over the lunar day as might indicate only a 2 or 3 minute a day deviation in the major rhythmic period from precise lunar-day frequency. These last deviations would have yielded a 2- or 3-hour phase shift, respectively.

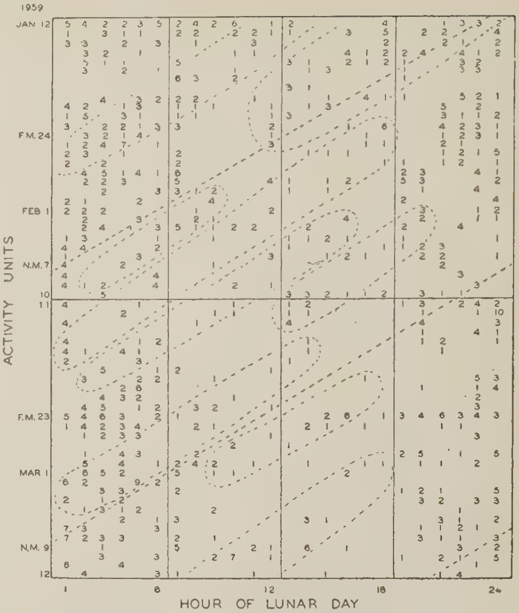

FIG. 1. Table including No. of activity periods of male white rat, kept in constant illumination and temperature, for each lunar-day hour during 60-day period.

Fig. 2, illustrating mean lunar-day cycles for each of 2 months (Jan. 12 to Feb. 10; Feb. 11 to Mar. 12), demonstrates reproducibility of these lunar-day cycles. The rat was more than 6 times as active at first lunar hour as at 11th one. The coefficient of correlation between comparable values for the 2 cycles was +0.70 ± 0.11. The general similarity of the average of these 2 cycles with the average 58-day lunar-day cycle for period, Nov. 15, 1955 through Jan. 11, 1956 published earlier, for a different rat, is evident from the calculated correlation coefficient of +0.56 ± 0.14 between corresponding lunar hourly values. Although an average solar-day cycle for

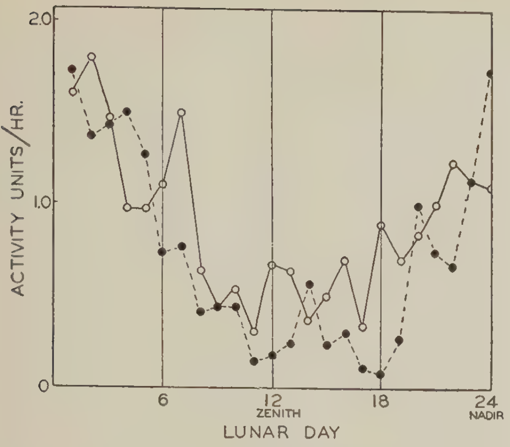

FIG. 2. Avg lunar-day cycle of running of the rat for 30-day period, Jan. 12 through Feb. 10 (solid line); for 30-day period, Feb. 11 through Mar. 12 (broken line).

the 60-day period was found with activity more than 50% higher at midnight than at 9 a.m., there was no significant correlation between corresponding hourly values of mean solar-daily cycles between first and second months. Hence, there was no sustained, reproducible fluctuation in activity of a solar-daily frequency comparable to that found for lunar day. The sharpness of lunar-day periodism of activity, however, enabled one to obtain some very suggestive information as to what underlies the failure to observe what might reasonably have been expected to be the predominant rhythmic period.

In Fig. 1 the broken diagonal straight lines indicate passage over lunar days of time of solar midnight. These lines, therefore, represent the slopes which would be paralleled by any patterns of locomotory activity possessing a solar-day frequency. A careful study of Fig. 1 reveals numerous clusters of data, especially evident during periods of relative inactivity in lunar day, which give indication of being of solar-day frequency. These either continue for a few days and terminate with no apparent explanation, or continue until engulfed in the overwhelming lunar-day periods of activity. It is possible that these transient solar-day periodisms, occurring at various hours of the day, were initiated by occasional times when food and water were replenished, or the weak illumination turned off, and that these transient daily cycles would not have become evident in wholly constant conditions. The 24-hour period itself was clearly not so imparted. Several of the more conspicuous of these solar-day recurring patterns are lightly outlined in the table.

Discussion. The foregoing results indicate the white rat possesses the capacity to display at the same time and same process rhythmic changes of 2 closely similar natural frequencies. These are the same periodisms which have been fully established as being derived continuously from external environment even in what physiologists have hitherto presumed constant conditions. The latter studies involved solar, lunar and annual rhythms of cellular oxidations(3,4,5,6).

Possession by the rat of a single autonomous periodism precisely the frequency of any single natural geophysical one is itself a highly improbable event; simultaneous presence of 2 autonomous periodisms both precisely those of major geophysical frequencies is even far less probable. Therefore, a reasonable hypothesis is that the 2 periodisms for spontaneous activity of the rat, differing in period by only 3½%, and exactly those of oxidative metabolism of numerous species of plants and animals, depend continuously upon force-fluctuations in the organism's environment effected by rotation of the earth relative to moon and sun.

Perhaps one of the most important suggestions from this study is that a behavior pattern, such as spontaneous locomotion, may be regulated through the same precise exogenous reference clock mechanism that is apparently responsible for regulation of periodisms in basal metabolic rate. This provides further evidence for a functional association between exogenous R oscillations and internal MI oscillations of the postulated(7) Responder-Mediator-Indicator mechanism of physiological-rhythm timing.

The results suggest that either of 2 exogenous metabolic periodisms, solar-day or lunar-day, depending upon other factors, is able to assume the dominant role in regulating the period of overt physiological cycles. This study, together with the foregoing one (1), also indicates that an overt cycle with period other than a natural one may or may not be present in the rat. In either case, however, the rat possesses the capacity for precise lunar-day and solar-day timing. Since the period of an overt rhythm which deviates from a natural one is known in the mammal (8) to be a function of constant illumination level, it is clear that such unnatural periods must be derived within the exogenously rhythmic organism in reaction with its environment. It has been pointed out(1,7) that all the evidence at hand is compatible with an hypothesis that the mechanism of their deviation involves the well-known phasing phenomenon, utilizing the demonstrated daily rhythm of sensitivity to light and temperature as phasing agents(9,10) to effect regular daily autophasing in constant conditions.

Summary. 1. A white rat displayed in constant low illumination a rhythm of spontaneous running of precise lunar-day frequency over 60-day study. This obviously resulted in accurate monthly recurrence of daily running patterns. 2. Activity was high during hours the moon was below the horizon, and low when above. Spontaneous running at first lunar hour was 6 times that at 11th hour. Correlation between average lunar-day cycles for 2 consecutive months was +0.70 ± 0.11, and mean cycle for each month was quite similar to earlier published lunar-day cycle in the rat. 3. Superimposed upon the dominant lunar-day periodism were minor, transient, solar-day patterns of locomotor activity, terminating spontaneously or through apparent submergence in the larger amplitude, lunar-day cycles. 4. Since these 2 basic periodisms in rat activity, solar-day and lunar-day, are the same frequencies as the widely, and probably universally, distributed exogenous metabolic periodisms, they are considered simple behavioral expressions of a fundamental exogenous "clock-system" from which regular physiological rhythms of other than natural frequencies, often present, may be derived by the organism.

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Autoradiographic Studies of Antimitotic Action of Maleuric Acid.* (24980)

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Maleuric acid (monomaleylurea) produces mitotic abnormalities and depresses mitotic index both in plant and animal cells(1).† In our study an analysis was made of mode of action of this compound on Ehrlich ascites tumor cells. By observing distribution of incorporated tritiated thymidine (T-H^3) in mitotic and interphasic cell populations using high resolution autoradiographic technics, it has been possible to show that administration of maleuric acid (MA) results in marked inhibition of the incorporation of T-H^3 into deoxyribonucleic acid (DNA) and, in addition, prevents progression of interphase cells into mitosis.

Methods. A sample of MA was obtained

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† LD_{50} in normal mice for single intraperitoneal injection was approximately 775 mg/kg and single daily doses of 150 mg/kg were tolerated for 8 days with no loss in weight or sign of toxicity.

from Naugatuck Chem. Division, U.S. Rubber Co., Naugatuck, Conn. On 4th day after transplantation of approximately 5 x 10^6 Ehrlich ascites tumor cells into C57 black mice averaging 20 g in weight, mice were injected intraperitoneally with 33 μc of T-H^3 (Schwartz Laboratories specific activity 360 mc/mM) in 0.1 ml saline. Single doses of 5 mg of MA in 0.5 ml saline were administered at times indicated below for individual experiments. Samples of tumor cells removed by peritoneal puncture were squashed with acetic dahlia for cytological examination or smeared for preparation of autoradiographs. The dried smears were fixed in absolute ethanol 5 minutes, brought to water through graded alcohol series, hydrolyzed in 1N HCl at 60°C 8 minutes, and treated with Feulgen stain. After appropriate bleaching and washing, the autoradiographic film was applied according to Taylor and McMaster(2) modification of stripping film technic. After 14-16 days of exposure, the autoradiographs were processed according to schedule of Taylor(3) and mounted in immersion oil. A nucleus was considered to be labeled if there were 6 or more grains above background level in the photographic emulsion over it. Since background generally amounted to 1 to 2 grains in an area as large as that overlying a nucleus, this criterion of labeling might tend to make our estimate of percentages of labeled cells slightly low. On the other hand, the level of labeling was such that the number of borderline cells was small in samples studied.

Results. As shown previously(1), MA produces marked cytoplasmic damage in all cells and chromosomal abnormalities characterized by clumping and stickiness (Fig. 1, normal cells; Fig. 2, treated cells). Photographs of typical results of labeling of untreated interphase and mitotic cells are shown in Figs. 3 to 5 and of cells treated with MA in Figs. 6 and 7. Usually between 500 and 600 interphases were counted for each sample with exception of samples obtained at later time intervals following injection of MA, when few tumor cells could be found in the smears. The smallest number of interphases observed for any sample was 88 cells. About 100 mitotic cells generally were counted, but at the later time intervals after treatment only a small number of mitoses could be observed.

In control experiments rapid incorporation of T-H^3 took place into interphase cells, approximately 40% of the cells being labeled in one hour after injection of this isotope (Fig. 8), the earliest time at which a sample was taken. Thenceforth, there was a slight increase of doubtful statistical significance in percentage of labeled cells up to 24 hours. The minimal period between time that exogenously supplied thymidine was incorporated into DNA and identification of labeled mitotic cell was 3 hours. In the 3-hour sample only 4 out of 194 (2.6%) mitotic cells were labeled, while at 4 hours the number rose to 26 out of 216 (12%). Maximal labeling of mitotic cells was attained at 12 hours. Actually, in one case, while only 2 of 96 mitotic cells were labeled at 3 hours, 53% of prophases, 7% of metaphases, and 3% of anatelophases were labeled at 4 hours. These values increased to 70, 29, and 4%, respectively, at 4.5 hours and 71, 53, and 54% at 6 hours. At 8 hours all phases were estimated to be labeled to approximately the same extent, 70-90%, no significant decrease being noted to 24 hours, the last period observed. Data from these control experiments appear to be in reasonable agreement with previously published results on incorporation of adenine-C^{14} into Ehrlich ascites cells(4).

Pretreatment of animals with MA had a profound effect on incorporation of T-H^3. When 5 mg of MA was injected 30 minutes *prior* to T-H^3 there was no incorporation into ascites tumor cells (Table I). In experiments in which 3- and 24-hour intervals elapsed between injection of MA and T-H^3 there was

TABLE I. Effect of Pretreatment with Maleuric Acid on Thymidine-H^3 Incorporation.*

	% of interphases with label			
		MA before T-H^3		
Hr after T-H^3	Control	30 min.	3 hr	24 hr
1	44	0		0
3	45	0	2	0
6	51	0	15	7
10		0	20	
12	55			
24	47		0	

* 5 mg MA in 0.5 ml saline given intraper.

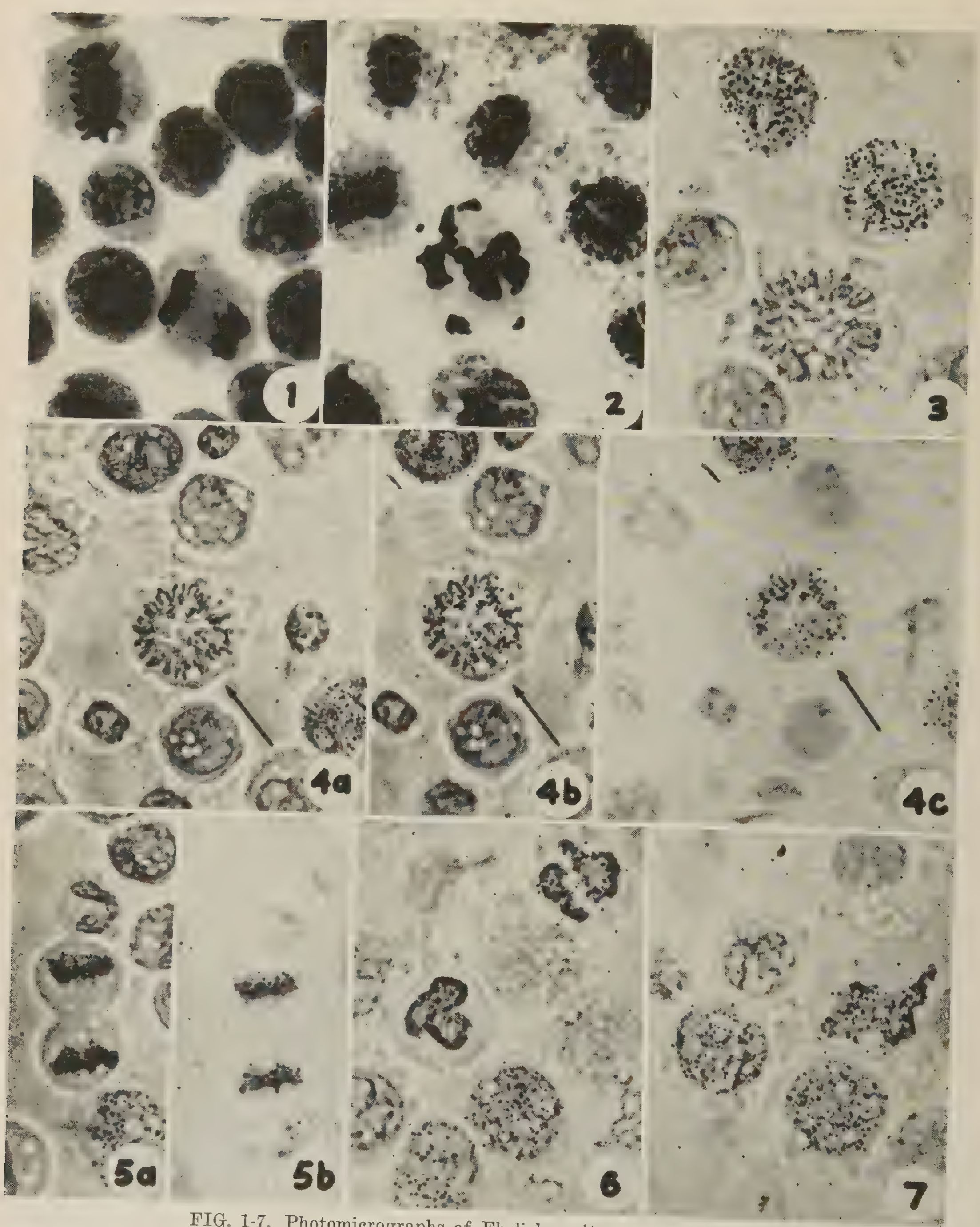

FIG. 1-7. Photomicrographs of Ehrlich ascites tumor. × 975.
FIG. 1. Untreated Ehrlich ascites tumor. Squash preparation stained with acetic dahlia.
FIG. 2. Tumor cells 3 hr after inj. of MA.

FIG. 3-7. Autoradiographs of Ehrlich ascites tumor stained by Feulgen reaction.
FIG. 3. Labeled interphase cells and unlabeled metaphase cell 3 hr after intraper. inj. of tritiated thymidine (autoradiograph; Feulgen stain; phase).
FIG. 4a-4c. Labeled metaphase cell (indicated by arrow) and both labeled and unlabeled interphase cells 10 hr after intraper. inj. of tritiated thymidine. (a) Phase contrast, showing

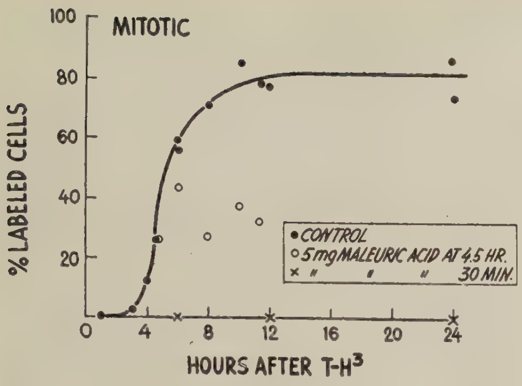

FIG. 8. Incorporation of T-H^3 into mitotic cells.

still a greatly reduced incorporation of label by comparison with controls.

When MA was injected 30 minutes or 4.5 hours *after* T-H^3, the percentage of labeled interphases decreased somewhat from control level during latter half of experiment. This decrease was slightly more pronounced when only 30 minutes elapsed between the 2 injections. Although number of cells in ascitic fluid was reduced greatly at 12- and 24-hour intervals, it is evident that MA did not cause greatly preferential destruction of labeled interphase cells; if lysis had occurred, it must have occurred at only slightly greater rates among labeled than unlabeled cells.

Observations on percentage of labeled mitotic cells clearly show that MA blocks entry of preprophase cells into mitosis. When injection of MA followed that of T-H^3 by 30 minutes, 47, 35, 26, and 27% of interphase cells were labeled at 3, 6, 12, and 24 hours, respectively, but not a single labeled mitotic figure was observed in samples studied. In the experiment in which MA was injected 4.5 hours after T-H^3, progression of labeled interphase cells into mitosis soon ceased even though labeled interphase cells were entering mitosis at a rapid rate (Fig. 8) at time of injection of MA. Although the accuracy of cell characterizations was reduced because of extensive damage to the mitotic figures, the impression was gained that a blocking of mitosis occurred at metaphase, probably related to observed stickiness and clumping. Thus, of 1511 untreated mitotic cells counted, the percentage distribution in prophase, metaphase and ana-telophase was 13, 55, and 32, respectively. In 326 cells counted after treatment with MA the distribution was 5, 82, and 13%.

Experiments to be published elsewhere showed that treatment with MA in doses comparable to those employed in present experiment in combination with other antitumor agents, while producing maximal cytological effects, caused no inhibition in ability of tumor cells to incorporate radioactive glutamine, glycine or lysine into cellular proteins at 3 hours after treatment.

Summary. The above results show that maleuric acid, at dosage level used, has at least 3 effects on cell cycle in Ehrlich ascites tumor cells. It prevents incorporation of tritiated thymidine into deoxyribonucleic acid, inhibits progression of premitotic cells into mitosis, and causes severe mitotic abnormalities which seem to result in a metaphase block.

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cells; (b) phase contrast in same field, showing developed photographic grains in field above cells; (c) same field, phase plate removed, showing developed photographic grains in film above cells.

FIG. 5a-5b. Labeled late anaphase cell 10 hr after inj. of tritiated thymidine. (a) Phase contrast, showing cell; (b) same field, phase plate removed, showing developed photographic grains in film above cell.

FIG. 6. Labeled and unlabeled abnormal mitotic cells at 8 hr after inj. of tritiated thymidine and 3.5 hr after inj. of MA.

FIG. 7. Labeled abnormal mitotic cell and labeled interphase cells at 8 hr after inj. of tritiated thymidine and 3.5 hr after inj. of MA.

Effect of Common Bile Duct Ligation on Lipoprotein Lipase Response in Dogs.*†‡ (24981)

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Decreased heparin-activated lipoprotein lipase (clearing factor) response has recently been reported in patients with obstructive jaundice(1,2,3). The etiology of this jaundice, however, varied greatly, being both intra- and extra-hepatic in origin, and subjects manifested varying degrees of inanition, acidosis or electrolyte imbalance. Consequently, to determine more precisely time course of events in obstructive jaundice, we completely ligated the common bile duct in normal dogs.

Material and methods. Subjects. In preliminary experiment, a female mongrel dog (Q) weighing 7.5 kg was subjected to complete ligation of common bile duct under pentobarbital sodium anesthesia. Subsequently, 6 female mongrel dogs (A-F), weighing 12 to 16 kg were placed on stock laboratory diets. After 3 week control period, complete ligation of common bile duct was performed on all 6 dogs under pentobarbital sodium anesthesia. *Lipoprotein lipase response.* Standardized *in vitro* method previously reported (3,4,5) for determining clearing factor or lipoprotein lipase concentration was employed. Control and 8 minute post-heparin blood samples were obtained from each dog at weekly intervals after overnight fast (at least 12 hours). Plasma was extracted by centrifugation at 0°C and frozen until used. *Evaluation of hepatic status.* At weekly intervals during 3 week control period, evaluation of hepatic status was performed on all 6 dogs. This included sulfobromphthalein (BSP) excretion, total and esterified cholesterol, serum bilirubin, icterus index, total proteins, albumin/globulin ratio, cephalin flocculation, thymol turbidity, gamma globulin turbidity, and alkaline phosphatase. Total serum lipids and lipid phosphorus were also determined on aliquots of control and post-heparin plasma samples.

Results. Table I demonstrates the effect of common bile duct ligation on lipoprotein lipase response in all dogs. Lipoprotein lipase response was *markedly decreased* in 4 out of 7 dogs one week after ligation of common bile duct, in fifth dog, 2 weeks, and in sixth and seventh dogs, 5 weeks after ligation. All 5 dogs who lived 4 weeks or more after surgery demonstrated *subsequent marked increase* in lipoprotein lipase response 4 to 10 weeks after ligation of common bile duct.

One dog (C) died one week postoperatively, death unrelated to experiment. A second dog (D) died 3½ weeks postoperatively with evidence of gross internal hemorrhage. Both dogs had markedly decreased lipoprotein lipase responses prior to death.

Lipoprotein lipase response was *generally inversely proportional* to total serum lipid, total serum cholesterol and lipid phosphorus levels. In Dog A, mean control lipoprotein lipase was 1.60, while mean control total lipids were 820 mg%. One week after common bile duct ligation, lipoprotein lipase response was markedly decreased to 0.76, while total serum lipids had doubled to 1820 mg %. Four weeks postoperatively, lipoprotein lipase response became distinctly elevated to 4.20, with abrupt drop in total serum lipids below control values to 505 mg %. Four days later this dog died. Postmortem findings included perforation of greatly dilated common bile duct with bile peritonitis and hemorrhage. The liver grossly demonstrated greatly dilated bile canaliculi.

In 4 dogs, B, E, F, and Q, *sustained* hyperlipemia and hypercholesterolemia were present for 2, 3, 5, and 5 weeks, respectively, be-

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‡ The authors acknowledge the courtesy of Dr. E. A. Hawk, Upjohn Co. in supplying heparin for this investigation. Ediol was kindly supplied by Mr. Robert E. Sullivan, Schenlabs Pharmaceuticals.

TABLE I. Effect of Common Bile Duct Ligation on Lipoprotein Lipase Response in Dogs.

Date samples drawn	Weeks after samples drawn	k†						
		Dog Q	Dog A	Dog B	Dog C	Dog D	Dog E	Dog F
12/ 8/58	0	Ligation						
15	1	1.26						
22	2							
29	3		1.63	1.51				
30					1.72	2.24	2.47	1.39
1/ 5/59	4		*		1.35	4.50		
8		1.25	1.94				1.55	1.44
12	5		1.33	2.31	1.97	1.83		
15		.48					1.22	1.34
19	6		Lig.‡	Lig.	Lig.	Lig.	Lig.	Lig.
22		.73						
26	7		.76	1.86	.21	.82		
29		.72			Exp.			1.47
2/ 2	8		.20	.24		.24		
5		.64					2.06	2.07
9	9		.62	.55		.14		
12		6.48					.51	2.23
13						Exp.		
16	10	2.00		1.44				
19			4.20				.21	2.31
23	11	1.98	Exp.	4.80				
24		Exp.‡						
26							.46	.21
3/ 2	12			2.39			.21	.23
9	13			3.96			.52	2.32
16	14			7.05				1.79
21				Exp.				
23	15						.42	1.08
25								Exp.
30	16						1.80	
4/ 5	17						2.24	
7							Exp.	

* Control period before common bile duct ligation.

† *k* is rate of decrease of optical density/hr. This is directly proportional to lipoprotein lipase (clearing factor) concentration in standardized *in vitro* assay method used(3,4,5).

‡ Exp. = Expired; Lig. = Ligation.

fore initial decrease in lipoprotein lipase response was observed. In 2 dogs, B and Q, total serum lipids reached levels as high as 2720 and 2880 mg %, respectively, serum total cholesterol being 430 and 422 mg %. These levels were accompanied by essentially *normal* lipoprotein lipase responses of 1.86 and 1.25, respectively. However, a significant ($P < .01$) negative regression of lipoprotein lipase response with increasing total serum lipids was found when all dogs were considered. Hyperbilirubinemia, *per se,* did not appear to influence lipoprotein lipase response. Perhaps the most remarkable elevation subsequent to biliary ligation occurred in serum alkaline phosphatase. In Dog A this reached 102.6 Bodansky units 3 weeks after ligation. Dog E rose to 126.3 units *one* week after ligation and 141.4 units 2 weeks after ligation. This observation, while not unique, is nevertheless quite striking when compared to humans with biliary obstruction. Lipid phosphorus rose with total lipids and cholesterol.

Percentage of cholesterol esters was variable, rising after biliary ligation in some instances, and dropping markedly preterminally in Dogs A, B, E, and F. Cholesterol/lipid phosphorus ratio demonstrated no trend. Neither of these parameters was related to lipoprotein lipase response. Total protein, A/G ratio, cephalin flocculation, and thymol turbidity were not very useful as indices of hepatic function or functional impairment. All dogs demonstrated 3^+ and 4^+ cephalin flocculation during *control* period, and in A, C, and E there was inversion of the A/G ratio during *control* period. This is generally found to occur in normal dogs. Forty-five minute BSP retention, however, was 4% or less during control period in all 6 dogs.

A pre-terminal increase in gamma globulin turbidity was probably concomitant with hepatic parenchymal cell damage. Fasting blood sugar levels were variable, Dog A demonstrating hyperglycemia (118 mg %) pre-terminally, and Dogs Q, E, and F demonstrating hypoglycemia (36, 24, and 45 mg %, respectively). *Decrease* in total lipids, total and esterified cholesterol, and lipid phosphorus generally accompanied the subsequent *increase* in lipoprotein lipase response, presumably reflecting severe hepatic parenchymal cell damage and biliary cirrhosis. Although prothrombin time, determined at intervals, was normal (10 to 12 sec.), some evidence of hemorrhage was generally present at postmortem.

Discussion. Hepatic perfusion experiments in rats(6), dogs and man(7) demonstrated inactivation or attenuation of lipoprotein lipase by the *normal* liver. The *markedly decreased* lipoprotein lipase response previously reported in patients with obstructive jaundice has been confirmed in dogs in this experiment. It is suggested that this decreased response is a reflection of abnormal lipid and lipoprotein lipase metabolism. Deficiency of lipoprotein lipase *per se* does not seem likely in view of the *distinctly increased* lipoprotein lipase response occurring subsequently. It is suggested that this increased response may be associated with development of biliary cirrhosis. This was grossly observed in liver of Dog E at postmortem. Microscopic examination of livers of these dogs will be reported. *Increased* lipoprotein lipase response in Laennec's cirrhosis has been previously reported(1,2,3,5), and we have recently reported *increased* response in post-necrotic cirrhosis(8,9). Partial hepatectomy in rats(10), carbon tetrachloride-induced hepatic necrosis in dogs(7) and in rats(10), and patients with Laennec's cirrhosis(7) demonstrated *increased* lipoprotein lipase response in hepatic perfusion studies. It is therefore hypothesized that *increased* lipoprotein lipase response observed subsequent to *decreased* response in dogs after common bile duct ligation represents loss of functional hepatic parenchymal tissue, chronic cholestasis resulting in *chronic* hepatic parenchymal cell damage leading to biliary cirrhosis.

Other possible pathogenetic mechanisms for *decreased* lipoprotein lipase response after common bile duct ligation include abnormalities in lipoproteins *per se*, formation of inhibitor(s) to lipoprotein lipase, or acceleration of *in vivo* inhibition or inactivation of lipoprotein lipase system by the liver. It is conceivable that chronic complete cholestasis may stimulate or activate hepatic parenchymal cells to *increase* their rate of inactivation of lipoprotein lipase. While elevation of serum lipids follows closely upon ligation of common bile duct, the fact that *decrease* in lipoprotein lipase response *lagged* hyperlipemia by as much as 5 weeks in 2 of the 7 dogs suggests that 2 *different* (but perhaps related) mechanisms are involved. Possible factors are being investigated.

Summary. 1. *Decreased* lipoprotein lipase response in obstructive jaundice in man has been confirmed by complete ligation of the common bile duct in dogs. 2. Subsequent *increased* lipoprotein lipase response in these dogs after chronic complete cholestasis of 4 to 10 weeks is attributed to development of biliary cirrhosis. 3. While lipoprotein lipase response was *generally inversely proportional* to total serum lipids, cholesterol, and lipid phosphorus levels, this relationship was by no means absolute. 4. Possible pathogenetic mechanisms for *decreased* lipoprotein lipase response in obstructive jaundice have been discussed. These mechanisms are being investigated.

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Production of Dietary Vit. K Deficiency in the Rat. (24982)

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It has been frequently stated that the rat, among other mammalian species, has no dietary requirement for Vit. K and that intestinal bacterial synthesis of this vitamin is adequate for normal blood coagulation(1). To produce Vit. K deficiency in the rat, sulfa drugs are usually administered to depress intestinal microbial synthesis(2), or flow of bile into the small intestine is arrested to diminish absorption of Vit. K(3). Prevention of coprophagy has recently been reported to result in Vit. K deficiency in rats fed a Vit. K-deficient diet(4), indicating that Vit. K microbially synthesized in the intestinal tract was not directly absorbed but obtained by recycling of the feces. Rats fed gamma-irradiated beef died from non-specific hemorrhages which appeared to be due to a severe but uncomplicated Vit. K deficiency(5). This diet was very low in Vit. K and produced feces which were not acceptable to the rat. The purpose of this report is to describe a purified diet which when fed to weanling male albino rats produced a severe dietary Vit. K deficiency, as indicated by hypoprothrombinemia and lethal hemorrhages, without recourse to drugs, surgery or coprophagy prevention.

Methods. Weanling male albino rats of Sprague-Dawley strain were housed individually in wire-bottomed cages in temperature-controlled laboratory. Feed and water were provided *ad lib*. Composition of the diet is given in Table I. Coprophagy was prevented according to the method of Barnes *et al.*(6). The percent coprophagy refers to the proportion of total fecal dry matter consumed by the rat. Whole plasma prothrombin times were determined on 10th day of experiment according to the method of Quick(7). Hemorrhagic deaths were characterized by one or more of the following symptoms at autopsy: testicular hemorrhage, subcutaneous bleeding, hemothorax, and nasal bleeding. Oral Vit. K supplementation was carried out by administering water solution of tetra sodium salt of 2-methyl-1, 4-naphthoquinone diphosphoric acid ester by a calibrated eye dropper, to each rat at time of feeding. There were 6 rats in each group and the experiment lasted 10 days.

Results. Table II gives results obtained. It can be seen that rats consuming the diet described in Table I had abnormally long plasma prothrombin times and suffered from lethal hemorrhage. When coprophagy was prevented, the condition was worsened considerably. In either case, 5 μg of Vit. K_3/rat daily completely protected the rats from both hypoprothrombinemia and hemorrhage. The rats grew well in all cases. Gain in body weight was about 7.5 g/rat/day for first 7 days of experiment. Coprophagy on this diet was about 30 to 40%, about half that observed when casein was used in place of the purified soy protein (Drackett assay protein).

Discussion. The rat's physiological requirement for Vit. K can be satisfied by dietary Vit. K and/or by intestinal microbial synthesis. In large part, the latter source depends on the extent of coprophagy practiced by the rat, since very little, if any, of microbial Vit. K was available when coprophagy was prevented, as shown by development of a severe deficiency in a very short time when coprophagy was prevented. The diet described in

TABLE I. Diet Used to Produce Vitamin K Deficiency in the Rat.

Ingredient	%
Sucrose	66.5
Purified soy protein (Drackett assay protein)	20.0
DL-methionine	.5
Vitaminized cerelose*	5.0
Salts mixture No. 446†	4.0
Wheat-germ oil	.5
Cod-liver oil	1.5
Glycerol	2.0
	100.0

* Supplied the following in mg/kg diet: thiamine hydrochloride, 10; riboflavin, 10; calcium pantothenate, 50; pyridoxine HCl, 5; nicotinic acid, 20; folic acid, 1; vit. B_{12}, 0.10; biotin, 0.10; choline chloride, 1000.

† Mameesh *et al.*(8).

TABLE II. Effect of Coprophagy Prevention and Menadione Supplementation on Hemorrhagic Syndrome and Hypoprothrombinemia of Vit. K Deficiency in the Rat (10 Days).

Diet	Oral supplement/rat/day	No. of hemorrhagic deaths	Plasma prothrombin time, sec. (mean and spread)	Coprophagy, %
Soy protein		1	32 (14- 66)	38
Idem	5 μg vit. K_3	0	14 (13- 18)	29
" , coprophagy prevented		4	92 (63-120)	
Idem	*Idem*	0	15 (14- 16)	

Table I, besides being very low in Vit. K, also appears to lead to low fecal consumption by the rat in comparison to usual casein-synthetic diet and therefore produces a severe Vit. K deficiency in 10 days. 5 μg of menadione/rat/day were adequate for maintenance of normal plasma prothrombin time and completely protected the rats from hemorrhage.

Summary. A diet is described which produces a severe Vit. K deficiency in the rat in less than 10 days. The deficiency was manifested by prolonged plasma prothrombin times and hemorrhages. Daily menadione supplementation protected rats from the deficiency. Coprophagy appeared to be the means by which Vit. K synthesized by the intestinal microflora becomes available to the rat.

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Convulsant Effects of Mikedimide (3,3, Methylethyl Glutarimide) in Monkeys.[*] (24983)

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Mikedimide[‡] (3,3, methylethyl glutarimide) has been recently introduced as a central analeptic with strong antagonism to barbiturates (1). Similarities in some of its actions in humans to those of pentamethylenetetrazole (Metrazol) (2-4) led to these investigations of its effects on a) normal, b) epileptic, and c) brain-operated non-epileptic monkeys.

Method. Tests were performed on 19 *Macaca mulatta* weighing 3-5 kg. In the first group of 11 monkeys, 5 had been made chronically epileptic by application of alumina cream to cerebral cortex(5) and 6 were unoperated normal controls. Mikedimide (0.5%) was injected intravenously "rapidly," *i.e.*, within 1-3 minutes at 1-4 ml/min, at intervals of 12 to 14 days. Clinical observations and electroencephalographic recordings were made for at least 30 minutes following injection; when clinical convulsive seizures occurred, sodium phenobarbital was promptly administered intravenously. Threshold convulsant doses were considered to be the least quantity which induced clinical convulsions. Thresholds for clinical convulsions to intramuscular Metrazol were also determined. In a second

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[†] St. Vincent's Hosp., N. Y. City.

[‡] Generously supplied by Panray Corp., N. Y. City.

group of 8 monkeys the effects of rapid intravenous injection of standard challenge test dose of 4 mg/kg Mikedimide were ascertained. Six of this non-epileptic group had been operated 4 to 13 months previously at which time various metals (tantalum, silicon, platinum, silver, cadmium or mercury) had been implanted in the motor cortex of both cerebral hemispheres; the remaining 2 monkeys were normal unoperated controls.

Results. Tonic clonic generalized clinical convulsions occurred in normal unoperated monkeys following rapid intravenous injection of 5-9 mg/kg Mikedimide (5, 5, 6, 6, 9, 9 mg/kg), while epileptic monkeys similarly treated had thresholds of 0.75-2.7 mg/kg, (0.75, 0.75, 1, 2, 2.7 mg/kg). In all cases, intravenous sodium luminal injected soon after onset of seizures promptly controlled them.

Thresholds for clinical convulsions of intramuscular Metrazol exceeded 40 mg/kg in all normal monkeys, whereas in epileptic monkeys threshold doses ranged from 12 to 24 mg/kg (12, 12, 16, 24, 24 mg/kg). Epileptic monkeys with lowest thresholds to intravenous Mikedimide had lowest thresholds also to intramuscular Metrazol.

None of the 6 brain-operated non-epileptic monkeys nor 2 additional normal monkeys showed clinical convulsions following rapid intravenous injection of a test dose of 4 mg/kg Mikedimide.

Discussion. In monkeys, rapid intravenous injection of Mikedimide in adequate dosage may induce clinical convulsions which can be readily controlled and terminated by intravenous barbiturates. Since threshold dosages for clinical convulsions were much lower for epileptic than for normal monkeys, use of an intermediate dose (4 mg/kg intravenously) should induce clinical convulsions in epileptic but not in normal monkeys. Mikedimide compares quite favorably with other agents similarly used such as intramuscular Metrazol, intramuscular and intravenous picrotoxin and intravenous semicarbazide (aminourea). Intravenous Mikedimide produces seizures usually within a minute following injection and threshold convulsant doses in epileptic monkeys seem to parallel those to intramuscular Metrazol. We believe, therefore, that Mikedimide may be used advantageously in evaluation of epileptic status of monkeys and should prove a valuable and reliable addition to the battery of pharmacologic tests developed for this purpose.

Summary. 1) Rapid intravenous injection in monkeys of adequate doses of Mikedimide (3, 3, methylethyl glutarimide) induced clinical convulsions which were readily controlled by intravenous barbiturates. 2) Thresholds for clinical convulsions were lower in epileptic than in normal monkeys. 3) A challenge dose (4 mg/kg) is suggested as one capable of producing clinical seizures in epileptic but not in non-epileptic monkeys.

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Isolation of Acid Soluble Nucleotides from Rabbit Intestinal Mucosa.* (24984)

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In studies on the action of drugs it was essential to isolate the acid soluble nucleotides in a manner that ratios of 5′-monophosphates to 5′-polyphosphate nucleotide derivatives approximate those found *in vivo*. Previously published procedures for isolation of acid soluble nucleotides from intestinal mucosa(1,2) necessitate several minutes between death of animal and isolation and acid denaturation of mucosa tissue. Thus, although these procedures gave adequate qualitative results, the quantitative aspects of analyses were suspected. Subsequently a procedure for isolation of acid soluble nucleotides of intestinal mucosa which completely eliminates any time lag between death of animal and acid fixing of mucosa tissue was developed.

Materials and methods. Young adult male albino rabbits were used. The animals were anesthetized with ether and the abdomen opened with long midline incision. Mucosa tissue was isolated by the following 2 procedures: a) The small intestine was ligated at a point approximating beginning of jejenum. The intestine was then severed at about 3 feet caudal to ligation. A 19 gauge hypodermic needle was inserted just inferior to point of ligation and 150 ml of ice-cold 3% perchloric acid slowly passed through lumen using 100 ml syringe. The acid-bathed segment of intestine was then excised and dropped into ice-cold 3% perchloric acid. After few minutes exposure to acid the intestine was removed and portions taken for histological examination. The remaining sections were blotted dry, cut open lengthwise, flattened out on blotting paper and the denatured mucosa removed by delicate scraping with forceps, Peyer's patches being excluded(3). The mucosa was then weighed and homogenized in 9 volumes of 3% perchloric acid. b) The identical intestinal region was excised without preliminary bathing with perchloric acid, cut lengthwise and flattened out on blotting paper. Intestinal contents were wiped away with absorbent cotton and water. The mucosa was removed in identical manner as in acid-fixed tissue, weighed and homogenized in 9 volumes of ice-cold 3% perchloric acid. The acid soluble nucleotides were isolated from perchloric extracts as previously reported(4). The solution containing concentrated acid soluble nucleotides was applied as spots to sheets of Whatman 3 MM paper and developed in first dimension with acid ammonium isobutyrate(5). Areas occupied by AMP, ADP and ATP wre cut out and quantitatively analysed as previously reported(4). Qualitative estimations of all components of acid extracts were made after chromatography in the second dimension with modified Markham and Smith solvent #3(6) [Aqueous $(NH_4)_2SO_4$ (564.8 g/liter) : water : isopropanol : 0.1 M versenate (78:19:2:1)].

Results. Qualitatively the same nucleotides were present regardless of whether or not the mucosa was bathed *in situ* with perchloric acid. These are the same as previously reported for rat(1) and rabbit(2) intestinal mucosa, *i.e.*, the acid extracts in both cases contained mono-, di, and triphosphates of adenosine, guanosine, cytidine and uridine together with inosine monophosphate, di-, triphosphopyridine nucleotide, several uridine diphosphate derivatives and a number of unidentified compounds containing adenine. Quantitatively the differences are very significant. Intestinal mucosa fixed *in situ* with perchloric acid consistently exhibited lower AMP and higher ADP and ATP level than mucosa tissue removed without preliminary perfusion (Table I). These results may be explained by the fact that perchloric acid denatures viable, well nurtured mucosa cells that have up to moment of fixation received ample supplies of oxygen and metabolites from the

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TABLE I. Effect of Perchloric Acid Fixation of Rabbit Intestine *In Situ* on Major Acid Soluble Adenosine Derivatives of the Mucosa.*

	μmoles/g mucosa wet wt		
Treatment	AMP	ADP	ATP
None	1.16 (.98, 1.34)	.32 (.39, .26)	.49 (.47, .51)
Perchlorate-treated	.10 (.16, .04)	.31 (.35, .27)	1.28 (.95, 1.60)

* All calculations are based on reported molar absorbance index for adenosine derivations, at 260 mμ(12). Values are avg of 2 experiments. Individual values are in parentheses. See *Materials and methods* for experimental details.

mesentery circulation. Thus concentration of 5′-mono and 5′-polyphosphate nucleotide derivates should approach that which occurs *in vivo*. These data are consistent with studies reported with tumor(7), lung(7), liver(8), and yeast(9), tissues in which most of the adenosine derivatives were present as 5′-polyphosphates. The same picture exists in mucosa when other nucleotide 5′-mono and 5′-polyphosphate nucleotides are compared. However in these cases quantitative values are unavailable since in *in situ* fixed tissues the amounts of guanosine-, cytidine-, and uridine-monophosphates present were so low that they could not always be detected. However, with mucosa tissue isolated without preliminary fixation, quantities of these 5′-mono-phosphate compounds were consistently observed. This is in keeping with observations of Siekevitz and Potter(10) who showed that non-adenosine 5′-mono- and 5′-polyphosphate nucleotide derivatives are in equilibrium with 5′-mono- and 5′-polyphosphate adenosine compounds. Impairment of high energy bond synthesis by production of cellular anoxia such as would occur in removal of intestine and subsequent isolation of respiring mucosa should produce a state of energy deficiency. This would be evident in a manner similar to that observed in respiring mitochondria treated with dinitrophenol or cyanide. In these cases(11) concentration of 5′-polyphosphate adenosine nucleotides decreased and AMP increased. Such was the case with mucosa tissue isolated without preliminary acid fixation (Table I). It is of interest that the morphology of mucosa tissue obtained by both procedures is normal (unpublished data). This may indicate that in non-perfused intestine ATP synthesis, although greatly diminished, continues at a rate fast enough to maintain cellular integrity.

Summary. A method is described which permits isolation of acid soluble nucleotides of the small intestinal mucosa. This procedure entails bathing intestine mucosa, *in situ*, with ice cold perchloric acid. In this manner, mucosa tissue is fixed while still in optimal nutritional state. The tissue so obtained gives a nucleotide pattern which both quantitatively and qualitatively resembles that of other normal well nurtured tissues.

Abbreviations used are: Adenosine mono-, di-, triphosphate, AMP, ADP, ATP.

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Effect of β-Aminopropionitrile (Lathyrus Factor) on Free Hydroxyproline, Proline and Glycine in Chick Embryo.* (24985)

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Chick embryos treated with beta-aminopropionitrile, the lathyrus factor, become stunted, develop gross skeletal deformities(1,2), histological changes in aorta, a startling degree of tissue fragility(3) and increased extractability of collagen(4). Alterations in behavior and properties of collagen of the lathyritic chick will be reported in detail elsewhere. Among acid-soluble, low molecular-weight constituents extractable from chick embryos are free amino acids including hydroxyproline(5). The latter is considered unique to collagen in the animal kingdom. Concentration of this amino acid rises in the brain of embryos treated with cortisone(6) although no explanation has been advanced. It is present in free form in negligible amounts in postnatal tissues, the currently held explanation being that proline is hydroxylated to hydroxyproline only after incorporation of the former into a polypeptide chain(7). This may not necessarily be the case in embryonic tissues, specifically those of the chick. There is a progressive decrease of free hydroxyproline in the chick brain with time and it is absent in adult birds(8). This study compares concentrations of total free amino acids, hydroxyproline, proline and glycine in brains and carcasses of normal and lathyritic chick embryos at 2 different periods of development. These 3 amino acids were chosen because of their particular prominence in amino acid constitution of collagen.

Methods. Fertile hen eggs were incubated under controlled conditions of temperature and humidity. On 4th day of incubation 0.32 mg (LD_{50}) of beta-aminopropionitrile fumarate (BAPN)[§] in 0.1 ml of saline was injected onto the chorio-allantoic membrane. Injected and non-injected batches of eggs were harvested on 10th and 17th day. Two separate and complete experiments were run for 10th day and 3 for 17-day embryo groups. Only living embryos showing typical stigmata of lathyrism (stunting, parrot beaks and bowed tibiae), were used. About 20 normal and 20 experimental embryos were processed in each experiment. They were decapitated while living, the carcasses rapidly weighed, chopped finely with scissors and homogenized with 2 volumes of ice cold 0.6 M perchloric acid. Brains were rapidly removed, weighed and plunged into liquid nitrogen. After pulverization in chilled steel mortar, the powder was homogenized with 2 volumes of 0.6 M perchloric acid for 2 minutes at about 5°C. In Exp. D half the brains were rapidly frozen in CO_2 snow instead of liquid nitrogen. Homogenates were cleared by centrifugation (59,000 x g, 30 minutes) and supernatant solutions were neutralized with KOH in the cold. Potassium perchlorate crystals were filtered off at 0°C and the resulting neutral extract was stored frozen. Analyses on whole extracts for glycine(9), hydroxyproline(10), proline(11), and α-amino acids with a ninhydrin method(12) were carried out before and after hydrolysis of the extracts. Hydrolysis was performed in 6 N hydrochloric acid in sealed tubes in oil bath at 138° for 3 hours. The results are recorded as "free" (result before hydrolysis) and "bound" (the difference between results before and after hydrolysis). Aliquots of extracts were freed of salt over a Dowex 50 column and examined by 2 dimensional paper chromatography in 2 different solvent systems(13 and Deffner personal

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[‡] The work was done during tenure of Established Investigatorship of Am. Heart Assn.

[§] Kindly supplied by Abbott Labs., Chicago.

TABLE I. Acid-Extractable Amino Acids of 10-Day Chick Embryos.

	μM/g wet wt							
	Brain				Carcass			
	Normal		Lathyritic		Normal		Lathyritic	
Exp. No.	A	B	A	B	A	B	A	B
Ninhydrin*								
Free	18.9	20.5	16.5	23.4	13.4	16.6	12.6	13.5
Bound	3.7	14.3	8.4	12.8	3.5	9.9	2.9	7.7
Hydroxyproline								
Free	.13	.17	.14	.20	.11	.14	.11	.13
Bound	.0	.02	.0	.04	.01	.05	.0	.04
Proline								
Free	.27	.36	.37	.43	.25	.28	.26	.26
Bound	.07	.14	.20	.16	.15	.13	.16	.08
Glycine								
Free	.51	.64	.53	.62	.62	.46	.59	.34
Bound	4.16	2.57	3.84	2.83	3.03	2.14	2.48	1.69
Pro/Hypro	2.1	2.1	2.6	2.1	2.3	2.0	2.4	2.0
Gly/Hypro	3.9	3.8	3.8	3.1	5.6	3.3	5.4	2.6

* Reported as μM leucine color equivalence.

communication) and stained with ninhydrin or isatin primarily to determine relative intensities of proline and hydroxyproline spots.

Results. Analytical data are tabulated in Tables I and II, the former referring to 10-day embryos and the latter to 17-day embryos. There are, at 10 days, no striking differences between extracts from normal and lathyritic embryos in any parameters examined, despite the fact that treated embryos (Table I) showed all morphological manifestations of lathyrism as well as greatly increased tissue fragility. The ratios of proline to hydroxyproline and glycine to hydroxyproline are essentially the same for normal and lathyritic animals in both brain and carcass. There is practically no bound hydroxyproline in extracts from any of the tissues, in contrast to proline, where there is a significant amount, and particularly to glycine where bound amino acid accounts for the bulk of total extracted. The free hydroxyproline, proline, and glycine concentrations in brain extracts are only slightly if at all higher than in extracts of the carcass.

Extracts from 17-day embryos, in sharp contrast to those described above, revealed significant differences in proportions of free hydroxyproline and proline in both brain and carcass (Table II). There is a significant increase of free hydroxyproline in the lathyritic brain over that in the normal, and a parallel decrease in free proline, the latter diminution also being clearly demonstrated in the carcass. In contrast there is an increase in amount of free glycine over 10-day embryo in both normal and lathyritic tissues. Thus, there is a reversal of the ratio of free proline to free hydroxyproline in the lathyritic animal whereas glycine to hydroxyproline ratio stayed essentially constant. Both features are manifested in the carcass as well as in the brain.

It is worthy of note that there is essentially no bound hydroxyproline extractable in perchloric acid as compared with proline and glycine, suggesting that there are little if any free small peptides or other complexes of hydroxyproline in the tissue.

Paper chromatograms run simultaneously on normal and lathyritic extracts and stained with isatin confirmed the rise in free hydroxyproline and the fall in proline.

Discussion. Reciprocal changes in free proline and hydroxyproline in the 17-day lathyritic chick embryo suggest an increased rate of conversion of the former to the latter rather than degradation of collagen to amino acids. The work of Mitoma *et al.*(14) strongly suggests that, in the chick embryo, hydroxylation of free proline does occur. This postulate leaves the sharp rise in free glycine, which comprises 30% of amino acid residues

TABLE II. Acid-Extractable Amino Acids of 17-Day Chick Embryos.

	μM/g wet wt							
	Brain						Carcass	
	Normal			Lathyritic			Normal	Lathyritic
Exp. No.	C	D	E	C	D	E	C	C
Ninhydrin*								
Free	24.3	25.7	18.5	20.8	22.8	17.6	16.5	13.8
Bound	17.8	14.8	13.1	15.1	15.0	14.8	15.2	12.2
Hydroxyproline								
Free	.31	.32	.22	.44	.42	.57	.33	.37
Bound	.02	.02	.0	.0	.0	.0	.12	.06
Proline								
Free	.41	.32	.52	.30	.27	.32	.44	.23
Bound	.20	.20	.17	.25	.25	.0	.45	.38
Glycine								
Free	.83	.95	.50	1.30	1.12	1.27	1.08	1.26
Bound	2.57	3.06	2.44	2.22	2.81	2.09	2.05	.84
Pro/Hypro	1.3	1.0	2.4	.68	.64	.56	1.3	.62
Gly/Hypro	2.7	3.0	2.3	3.0	2.7	2.2	3.3	3.4

* Reported as μM leucine color equivalence.

of collagen, unexplained. If these changes do represent collagen degradation, proline must be rapidly reutilized in other pathways in contrast to the other 2 amino acids.

Since the amino acid changes are first observed long after structural changes are well defined, it may be concluded (a) that the former are due to a lathyrus factor effect on mechanisms which are not detectable until late in maturation of the embryo and (b) that the agent may exert its deforming effect along other pathways. The fact that similar free hydroxyproline increases unaccompanied by skeletal deformities have been noted after cortisone administration favors these hypotheses.

Studies of the free amino acid patterns in similarly deformed embryos produced with 6-aminonicotinamide(15) or semicarbazide (16) will be of interest.

Summary. Analyses of brain and carcass of normal and lathyritic chick embryos for free hydroxyproline, proline, glycine, and total ninhydrin reactive material are reported. At 10 days development, 6 days after BAPN administration, no obvious alterations in these parameters were found although embryos were grossly malformed. At 17 days of development, 13 days after treatment, a reversal in ratios of proline to hydroxyproline was found; proline concentration fell, and that of hydroxyproline rose in both brain and carcass of lathyritic embryos as compared to the normals. A rise in free glycine concentration resulted in unchanged ratio of glycine to hydroxyproline.

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Inhibition of Steroid Mediated Pyridine Nucleotide Transhydrogenase and 17 β Hydroxysteroid Dehydrogenase by 2′Adenylic Acid.* (24986)

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Kaplan *et al.*,(1) have shown that 2′-adenylic acid (2′AMP) increases the rate of bacterial transhydrogenase. Inhibition by 2′AMP of placental estradiol-17β mediated pyridine nucleotide transhydrogenase and 17-β hydroxysteroid dehydrogenase has been demonstrated(2). At pH 6.8, 17-β hydroxysteroid dehydrogenase was markedly inhibited with respect to oxidation of estradiol by DPN and less affected with respect to reduction of estrone by TPNH while reactions of estradiol with TPN and of estrone with DPNH were unaffected. Evidence will be presented regarding the specificity and mechanism of this inhibition.

Materials and methods. Transhydrogenase preparations. 20% placental homogenate was prepared from normal term human placenta in 0.25 M sucrose in Potter homogenizer by rotation of teflon pestle for 1 minute. After straining thru nylon gauze the supernatant fraction was centrifuged 45 minutes at 50,000 x g and 2°C. The supernatant was then dialyzed 12 hours against 0.005 M phosphate buffer pH 7.0. This preparation is hereafter referred to as dialyzed fraction. It contained isocitric dehydrogenase-TPN, 17β-hydroxysteroid dehydrogenase, and traces of TPN. For study of the effect of 2′AMP on acetyl pyridine DPN transhydrogenation and dehydrogenation the dialyzed fraction was freed of nucleotides by addition of equal volume of saturated ammonium sulfate. The precipitate was redissolved in ¼ volume of distilled water and dialyzed 2 hours in cold room against 0.005 M phosphate buffer. Both dialyzed and ammonium sulfate purified preparations were stored at −20°C. DPN and TPN were obtained from Sigma. Acetyl pyridine DPN was obtained from Pabst. Deoxyadenylic acid, Deoxyguanilic acid, Uridine 5′monophosphate, Guanosine 5′Triphosphate, Adenosine 3′monophosphate were obtained from California Corp. for Biochemical Research. Cytidine 2′3′cyclic phosphate and Uridine 2′3′cyclic phosphate were obtained from Schwarz Labs. When Ba salts were obtained, these were converted to sodium salts. 2′AMP was obtained from Schwarz Labs. and purified by chromatography on Dowex-1 in the formate form. The product was recrystallized from water. 2′5′diphosphoadenosine and nicotinamide mononucleotide were prepared by hydrolysis of TPN by venom diesterase according to Wang, Shuster and Kaplan(3). 2′Inosinic monophosphate (2′IMP) was prepared by modification of that used by Shuster and Kaplan(4) and purified by chromatography on Dowex-1 in the formate form. Enzymatic assay for transhydrogenation between TPNH and DPN by measurement of α-ketoglutarate produced from isocitrate has been described (5). 17β-hydroxysteroid dehydrogenase was measured at same concentration of estradiol-17β (3 x 10^{-6} M) as that employed in measurement of transhydrogenase. This was accomplished by measurement of formation of reduced pyridine nucleotide in an Aminco-Bowman spectrophotofluorimeter. Sensitivity of this instrument permitted reaction rates to be followed when only catalytic quantities of steroid were present. The incubation system consisted of 0.006 M phosphate buffer pH 6.8, Versene 0.001 M, estradiol-17β 3 x 10^{-6} M, DPN 3 x 10^{-4} M, $MgCl_2$ 0.006 M and 0.1 ml dialyzed enzyme. The reaction was started by addition of enzyme to the incubation mixture at room temperature and the linear reaction rate determined. The exciting wave length was 340 mμ and the fluorescent wave length 450 mμ. Assays for dehydrogenase and transhydrogenase could be duplicated within 5%.

Results. Inhibition of transhydrogenase by 2′AMP. Table I indicates that 2′AMP inhib-

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TABLE I. Inhibition of 17-β Hydroxysteroid Dehydrogenase and Estradiol Mediated Transhydrogenase by Nucleotides.

		% inhibition	
Substance	Conc., M	Dehydrogenase	Transhydrogenase
2'AMP	1×10^{-3}	100	95
	1×10^{-4}	99	54
	1×10^{-5}	65	0
	1×10^{-6}	6	
2'IMP	1×10^{-3}	88	97
	1×10^{-4}	82	
	2×10^{-5}	38	36
5'AMP	1×10^{-3}	50	
	1×10^{-4}	25	0
3'AMP	1×10^{-3}	33	20
	1×10^{-4}	0	16
5'UMP	1×10^{-3}	0	24
	1×10^{-4}	0	16
NMN	1×10^{-3}	40	17
	5×10^{-5}	0	0
2'5'ADP	1×10^{-3}	65	0
	5×10^{-5}	58	0

5'Deoxyguanylic, 5'deoxyadenylic acid, cytidine 2'3'cyclic monophosphate, uridine 2'3'cyclic monophosphate, 5'IMP and 5'guanosine triphosphate showed no inhibition of either enzyme at 10^{-3} M.

its dehydrogenase and transhydrogenase activity to a significant degree. The inhibition of dehydrogenase was usually somewhat more than that of transhydrogenase activity. 2′IMP also showed significant inhibition of both activities. 2′5′ADP showed significant inhibition of dehydrogenase activity but no measurable inhibition of transhydrogenation. The 5′AMP and 3′AMP were far less effective inhibitors than 2′AMP. A variety of other nucleotides showed little inhibition of either system.

Effect of 2′AMP on dehydrogenation and transhydrogenation involving acetyl pyridine DPN. Rate of transhydrogenation between DPNH and acetyl pyridine DPN (APDPN) was measured spectrophotometrically at 400 mμ. The incubation mixture consisted of 0.1 M Tris buffer pH 7.4, DPNH 0.0002 M, APDPN 0.001 M, estradiol-17β 6 x 10^{-6} M, and 0.10 ml ammonium sulfate purified placental enzyme which contained approximately 0.1 mg protein. In accordance with observations of Talalay(6) transhydrogenation between DPNH and APDPN was readily measured at 400 mμ because of negligible adsorption of the former substance. Transhydrogenation was inhibited completely at 10^{-3} M 2′AMP and 65% inhibited at 10^{-4} M. No inhibition was observed at 10^{-5} M. Oxidation of estradiol-17β by APDPN was studied at 6 x 10^{-6} M steroid, similar to that employed in the study of transhydrogenation to APDPN. The reaction was measured fluorimetrically with excitation wave length 400 mμ and fluorescence wave length 475 mμ. The incubation mixture was similar to that used for study of DPN except for the indicated steroid concentration. 2′AMP caused 60, 45 and 25% inhibition at concentrations of 1 x 10^{-4}, 1 x 10^{-5}, and 5 x 10^{-6} M respectively.

Attempts to reverse 2′inhibition. Variation of estradiol, Mg^{++}, or phosphate concentrations over a 10-fold range, or doubling DPN concentration had no effect on 2′AMP inhibition of dehydrogenation of estradiol by DPN or transhydrogenation between TPNH and DPN. Addition of nicotinamide, cysteine, or pyrophosphate did not alter inhibition in either the dehydrogenation or transhydrogenation systems.

Discussion. These observations indicate that the estrogen sensitive enzyme system in human placenta is inhibited by low levels of 2′AMP. Inhibition appears to depend on the 2′ structure since 2′IMP is also an effective inhibitor while 5′IMP inhibits neither reaction. This inhibition appears fairly specific since a variety of other nucleotides are considerably less effective. Inhibition of transhydrogenation is consistently less than that for dehydrogenation. Studies by Talalay *et al.*, (6) indicate that transhydrogenation between TPNH and DPN is very dependent on TPN concentration. The lesser inhibition of transhydrogenation may be due to competition of TPN and the 2′AMP for the same site on the enzyme. 2′AMP is known to inhibit TPN enzyme systems competitively at somewhat higher concentrations than used in these studies(7). However, 2′AMP is an effective inhibitor when transhydrogenation involves DPNH and APDPN in absence of TPN. TPN completely inhibits transhydrogenase at 10^{-5} M(6), 2′AMP is a less effective inhibitor.

Appropriate hydrolysis of TPN yields nicotinamide mononucleotide, a poor inhibitor of either dehydrogenase or transhydrogenase, and 2′5′ diphosphoadenosine, a substance with considerable activity to inhibit dehydrogenase but no demonstrable effect on transhydrogenation. This latter lack of inhibition is probably due to easy displacement from the active site by TPN. These data are consistent with the identity of 17β hydroxysteroid dehydrogenase and steroid mediated pyridine nucleotide transhydrogenase proposed by Talalay and Williams-Ashman(8). If these 2 enzyme activities do not reside in the same molecule the similarity in their inhibition by 2′AMP is remarkable.

Evaluation of the role of steroid mediated transhydrogenation in slices of estrogen sensitive target organs is now in progress with the aid of 2′AMP as inhibitor of this process.

Summary. 2′AMP inhibits placental 17β hydroxysteroid dehydrogenase and steroid mediated pyridine nucleotide transhydrogenase. This inhibition is due to the 2′ structure and occurs in transhydrogenation which does not involve TPN.

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Transient Secretion of Phosphate in the Mammalian Kidney* (24987)

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Even though phosphate secretion into the renal tubule has been invoked to account for a wide variety of data(1-6), such a process has never been demonstrated reproducibly in the dog. Secretion of phosphate, demonstrated reproducibly by clearance technic, has been reported in cats and chickens. Taugner(4) reported net secretion by the cat under conditions of loading with various organic phosphates esters *i.e.*, fructose 1,6-di-phosphate, phosphoglyceric acid, and glycerophosphate. Davidson reported net secretion in the chicken after intravenous administration of parathormone(6). Such findings suggest that secretion of phosphate might occur in all mammals under certain conditions. A comprehensive working hypothesis would require that phosphate is excreted *via* glomerular filtration, tubular reabsorption, and tubular secretion. If rate of reabsorption exceeded that of secretion under steady-state conditions of phosphate loading, net secretion could not be demonstrated by conventional clearance technic. On the other hand, a secretory mechanism might respond more rapidly to transient increase of concentration of serum phosphate than would a reabsorptive mechanism. If such an event could occur, the transient excretion of phosphate would be greater than that of creatinine or of any "glomerular substance." Such considerations prompted an exploration of transient renal response to close-arterial instantaneous injection of phosphate into the renal artery. The technic used to study transient responses of dog kidney was developed by Chinard(7).

Methods. Nembutalized dogs were given priming and maintenance infusions of 1 molar mannitol containing creatinine, KH_2PO_4, and NaH_2PO_4 in amounts sufficient to maintain high steady-state levels of urine flow, and to

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‡ Work done during tenure of Established Investigatorship of Am. Heart Assn.

maintain constant levels of creatinine (1 m M/L), and of phosphate (3 m M/L) in plasma. After 40-60 minutes of maintenance infusion, urine flow reached values of 8-12 ml/minute/kidney. After collection of 4 or 5 such preliminary periods *via* catheters in both ureters, one ml of a solution of 0.5 molar creatinine and 0.5 molar NaH_2PO_4 was injected *via* flank incision, through the aorta, directly into the renal artery(7). In some experiments, known amounts of radioactive phosphate (P^{32}) were included in the close-arterial injection. Following injection, urine was collected at 6-18 seconds until 20-30 such aliquots were obtained. A specially designed fractional collector§ facilitated sampling of urine. Analytical technics employed were that of Folin for creatinine(8); that of Gomori for inorganic phosphorus(9); and that of Geiger-Muller counter for P^{32}. Amount of substance excreted by the 'experimental' kidney following a single circulation of injected material through that kidney, (ΔE), was determined from the expression, $\Delta E = E_e - E_c$, where E_e = amount of phosphate in urine of injected side, and E_c = amount of phosphate in simultaneously collected urine of the non-injected side. Like Chinard(7), we assumed that the subtraction would correct for re-circulation, provided that blood flow, filtration rate, and tubular activity of the 'non-injected' kidney were equal to those of the injected kidney. Precautions were that rate of urine flow and of phosphate excretion of each kidney be almost equal (less than 10% discrepancy) throughout the experiment. An identical formulation was applied to raw data on creatinine, the 'glomerular' substance. Values of ΔE for phosphate and creatinine were normalized with respect to the determined amount of each substance (I) in injection solution by the ratio $\Delta E/I$. Thus $\Delta E/I$, the fundamental parameter used herein, was the increment of excretion of a given substance/unit injected/one circuit of that substance through the kidney. When $\Delta E/I$ of one substance is compared to that of another injected simultaneously, their relationship with respect to raw data on concentration, or with respect to amount will be the same, since urine volume term will cancel. Consequently we plotted values of $\Delta E/I$ in terms of concentration rather than amounts.

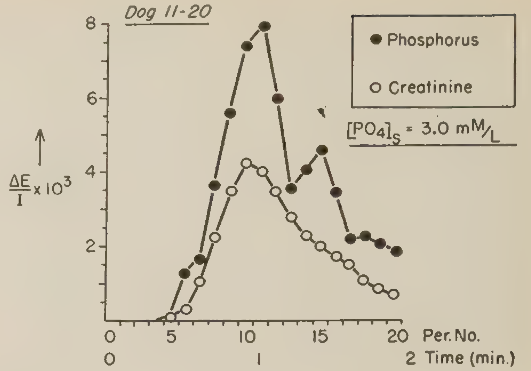

FIG. 1. Normalized incremental concentration of phosphate and of creatinine *vs* time (or period number). Phosphate values of those chemically determined(9).

Results. Fig. 1 is plot of values of $\Delta E/I$ *vs.* time in a representative experiment. The normalized increment of phosphate excretion/one circulation of injected material ($\Delta E/I$) was greater than that of creatinine for 14 out of 15 post-injection periods for 2 minutes. Peak value of $\Delta E/I$ for phosphate was 8.0 x 10^{-3}, while that of creatinine was 4.2 x 10^{-3}. This means that the amount of phosphate added to urine was almost twice that expected if phosphate had been excreted exactly as had creatinine.

Similar phenomena were found in 7 other experiments, wherein phosphate levels of serum had been kept at levels of 2.5-4.0 m M/L for 40-60 minutes prior to instantaneous injection. Transient excretion of phosphate greater than that of creatinine was found during both upsweep and downsweep of curves similar to that of the figure. Identity of incremental excretions of phosphate and creatinine was observed most frequently during periods of peak excretion of both substances. Patterns of P^{32} excretion were closely similar to those of non-radioactive phosphorus.

Since creatinine is excreted in a manner identical to that of other glomerular substances under present experimental conditions (7), the observed excess of phosphate over creatinine excretion must represent a minimal amount of phosphate secreted from interstitial fluid to tubular lumen. This is compatible with the concept requiring that tubular handling of phosphate includes reabsorptive and secretory processes.

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§ Fraction collector for urine designed and built by Mr. John Armand, Johns Hopkins Univ. School of Medicine.

Summary. Transient renal responses to close arterial instantaneous injections of NaH_2PO_4 and creatinine were studied in phosphate-loaded dogs. The increment of phosphate excretion per unit injected per one circulation of injected substance was greater than that of creatinine. Such a finding, interpreted as net minimal secretion of phosphate into the renal tubule, was found in 8 consecutive experiments.

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Viremia in ECHO Type 6 Virus Infection.* (24988)

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Extensive use of improved tissue culture procedures in the study of infectious diseases has prompted the recognition of an increasing number of cytopathogenic agents with characteristics of ECHO (Enteric Cytopathogenic Human Orphan) viruses(1). While the importance and etiologic role of these agents in human disease require further clarification, several already have been associated with outbreaks of non-paralytic illnesses, lymphocytic meningitis and infantile diarrhea. Their detection has been accomplished more often in stools than in pharyngeal secretions and cerebrospinal fluid of clinically ill individuals. However, limited data are available(2,3,4) on occurrence of viremia in ECHO virus infections. The present report describes isolation of an agent, antigenically indistinguishable from prototype ECHO 6 virus, from blood of a 7 year old female prior to fatal termination of a paralytic illness not associated with a stiff neck or other signs of meningeal involvement. Results of serologic studies of familial associates also are included.

Methods. Primary human amnion cell cultures prepared by modified Lahelle procedure (5) were utilized for both virus isolation and neutralization studies. The trypsin dispersed cells were grown in medium consisting of 0.5% lactalbumin enzymatic hydrolysate in Hanks solution and supplemented with 15% heated (56°C for 30 minutes) horse serum. After 5-10 days incubation at 36.5°C the monolayer cultures were washed once with Hanks solution, then 1 ml of lactalbumin maintenance medium containing 2% heated calf serum was added/tube. The latter medium also was used for propagation and maintenance of monkey kidney tube cultures prepared by modified Youngner technic(6). In virus isolation tests, 0.2 ml of each specimen were inoculated into 6 tubes each of human amnion and monkey kidney cell cultures. Certain specimens and tissue culture fluids also were tested by intraperitoneal (0.03 ml) and intracerebral (0.02 ml) inoculation of suckling mice less than 24 hours old. Throat swabs were eluted into 3 ml portions of Hanks solution prepared with 500 *u* penicillin, 250 μg streptomycin and 40 *u* mycostatin/ml;

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TABLE I. Summary of Clinical Features and Virus Isolation Attempts.

Day of illness	Clinical features	Specimen	Virus isolation attempts TC	Virus isolation attempts SM
1	Abrupt onset, muscular weakness lower extremities, no fever	None		
2	Fever, flaccid paralysis, abdominal reflexes diminished, deep tendon and gag reflexes absent	Throat swab	Neg.	Neg.
		Stool	"	"
3*	Progressively worse	Throat swab	Neg.	Neg.
		Stool	"	"
		Blood	Pos.	"
		CSF	Neg.	ND
4	Same			
5	" + pneumonic infiltration			
7	Expired	Sigmoid colon and contents	Neg.	Neg.
		Ileum	"	ND
		Mesenteric lymph nodes	"	"
		Cervical and lumbar cord	"	"
		Medulla and pons	"	"
		Cerebral cortex	"	"

TC = tissue culture; SM = suckling mice; ND = not done.
* CSF = 3 cells/cmm (all lymphocytes); total protein 18 mg %.

Hanks solution containing 2000 *u* penicillin and 1000 μg streptomycin/ml was used in preparing 20% suspensions of stool and post-mortem specimens, clarified by centrifugation at 5800 x g for 45 minutes prior to use. Sera employed in neutralization tests were inactivated at 56°C for 30 minutes. Neutralization mixtures prepared with equal volumes of serially diluted serum and constant amounts of virus were incubated at room temperature for 1 hour before inoculation; 0.2 ml of each mixture being added to each of 3 tube cultures.

Results. Correlation of clinical course with time of collection of specimens and with results of virus isolation attempts is shown in Table I. A single whole blood specimen taken 3 days after onset of illness and on 2nd day of paralysis produced specific cytopathogenic changes in both human amnion and monkey kidney cell cultures. The degenerative changes first became evident 8 and 6 days, respectively, after inoculation of the 2 host systems and complete destruction of cellular growth occurred within additional 72 hours in each instance. Subsequent titrations performed in 2 lots of amnion cultures revealed approximately 65 TCD_{50} of virus/ml of whole blood. Presence of virus also was demonstrated in patient's serum obtained on 3rd day of illness and stored at –20°C, for 3 weeks, before inoculation into different lots of amnion and kidney cultures.

All pharyngeal, stool, cerebrospinal fluid and post-mortem specimens failed to yield cytopathogenic agents after 2 consecutive passages in each culture system. Decreasing the concentration of inocula in cultures gave no evidence that "blocking" or "interference" effect was responsible for these negative results. The pharyngeal and stool materials as well as virus containing whole blood produced no demonstrable illness in suckling mice during 2 consecutive passages made at 10-day intervals. Likewise, virus-containing culture fluids from 4th amnion and kidney cell passages ($10^{-6.7}$ and $10^{-6.4}$, TCD_{50}/ml respectively) of blood isolate failed to produce signs of illness in suckling mice. In the latter tests virus could be recovered from pooled brain and muscle of mice 2 but not 12 days after inoculation.

Whole blood and serum isolates were identified as ECHO type 6 virus in neutralization tests performed with 1:100 dilution of first passage culture fluids and 1:10 dilution of prototype ECHO 6 (D'Amori strain) rabbit antiserum. In quantitative homologous and heterologous neutralization tests performed with the prototype antiserum and blood isolate antiserum prepared in rabbits (Table II), no significant antigenic difference was found

TABLE II. Titration of Antisera against ECHO 6 Viruses.

Rabbit antiserum	Blood isolate			Prototype 6 (D'Amori)		
	32*	100	500	32	100	500
Prototype ECHO 6	320†	320	160	320	160	160
Blood isolate	640	640	640	640	640	320

* TCD_{50} of virus used in tests.

† Reciprocal of serum dilution neutralizing indicated amount of virus.

between the 2 virus strains when indicated amounts of virus were employed. Relationship between homologous and heterologous serum titers at any given virus concentration was constant regardless of whether the tests were read on 4th or 8th day, although serum titers usually were 2-fold lower at the latter time. Neutralizing antibodies were not demonstrable in virus-containing patient's serum.

Three months after patient's fatal illness, sera† were obtained from 6 household associates, aged 3 to 34 years, and tested for neutralizing antibodies to the blood isolate and prototype 6 strains. Only one familial contact, aged 14 years, possessed serum antibodies (1:8 titer) to both virus strains. This finding could indicate a present or past subclinical infection with ECHO 6 virus.

Discussion. Information obtained from the study of this case indicates that systemic infection with ECHO type 6 virus may occur without frank meningeal involvement and suggests that viremia may be a significant part of pathogenesis of such infections. Detection of a viremic stage at least 2 days after development of definitive paralysis is of particular interest in view of absence of demonstrable virus in pharyngeal secretions and intestinal tract. This suggests that if the intestinal tract was the locus of initial infection, as currently purported for ECHO viruses, then viral multiplication was minimal at this site and probably occurred relatively early in the incubation period. The possibility also exists that virus multiplication in tissues other than those tested may have contributed to the late viremia.

Viremia in pathogenesis of ECHO 6 infection has not been previously recorded. However, an outbreak of exanthem disease in Pittsburgh, Neva and Zuffante(*2*) isolated virus from blood of one patient on second day of illness. This agent since has been identified as ECHO 16 and is related to if not identical with strains recovered earlier from similar cases in Boston(7). Brief reference was made by Sabin *et al.*(3) to detection of ECHO type 9 viremia prior to onset of clinical manifestations. No details were given but they indicated that viremia rarely was demonstrable within 24 hours after onset of febrile period. Observations by Yoshioka and Horstmann (4) also indicate that type 9 viremia is more likely to precede clinical disease.

It is reasonable to expect that further search will reveal presence of viremia in systemic infections with other ECHO viruses. Clarification of its role in pathogenesis of various clinical manifestations will necessitate more frequent inclusion of multiple blood specimens in virus isolation studies of suspected ECHO infections.

Summary. ECHO type 6 virus was isolated from whole blood and serum obtained 3 days after onset and on 2nd day of paralysis of fatal illness in which there were no meningeal signs. Virus could not be demonstrated in pharyngeal secretions, stools, CSF and postmortem materials. Neutralizing antibodies were not present in the virus-containing serum.

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† We are grateful to Dr. R. E. Harper, Colbert County Health Officer, for procuring these sera for us.

Effect of Temperature on Antibody Synthesis in the Reptile, *Dipsosaurus dorsalis*.* (24989)

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Although studies of antibody synthesis in mammals and birds have been confined to relatively few species, considerably more is known about the process in these 2 classes than about antibody synthesis in lower vertebrates. That lower classes of vertebrates are poikilothermic makes these animals logical choices for studies involving effects of temperature on antibody synthesis. In addition, a comparative study of antibody synthesis among various classes of vertebrates promises to provide much interesting information(1). The work of others has established that antibodies can be produced by fish(1) and frogs (2) in response to injection of particulate antigens, and in both an effect of environmental temperature on the process was noted. Evidence that reptiles are capable of forming antibodies is seen in experiments of Downs(3) on anaphylaxis in the turtle. The present paper reports studies on antibody synthesis at 25°C and 35°C in the reptile, *Dipsosaurus dorsalis* (desert iguana) in response to injections of formalin-killed *Salmonella typhosa* antigen.

Materials and methods. Forty adult specimens of *D. dorsalis* captured in Riverside County, Calif.(4), were maintained in laboratory on a diet of lettuce and mealworms, *ad lib.* Immunization was initiated within 3 weeks after capture to insure that animals would remain in good physical condition for duration of experiment. The antigen employed was a suspension of *Salmonella typhosa* prepared from 13 liters of BHI broth culture. After 24 hr of growth at 37°C, 0.3% formalin was added. Cultures remained at room temperature for 3 hr followed by overnight refrigeration. The cells were centrifuged and suspended in 500 ml of saline containing 0.3% formalin. To provide antigen for immunization, this stock was centrifuged and the cells diluted with sterile saline to optical density of 0.824 (540 mμ). During first week of immunization all 40 animals received 0.2 ml of antigen in subcutaneous tissue of thigh. Such injections were made daily in alternate hind legs for the first 3 days of the week. During following week 0.2 ml of antigen was given intraperitoneally on first 3 days of week, total 6 injections. During immunization, some animals were maintained at 25°C and some at 35°C. After completion of the 2 week immunization period, the animals were held for 7 or 14 days before bleeding as shown in Table I. The animals were bled by cardiac puncture and blood expelled into centrifuge tubes where it clotted. To measure agglutinin content of serum, the test was set up as in(5) except that 0.2 ml quantities of serum and antigen were used. The antigen was prepared from stock (H) suspension employed for immunization by diluting it to correspond to tube 3 of McFarland barium sulfate standard. The agglutination was read after 2 hours in 37°C bath.

Results. A 2 week immunization period was sufficient to produce good antibody titers in animals maintained at 35°C (Table I). Agglutinations were of the H variety and were strong and easy to read. There appeared no significant difference in groups maintained at 35°C for 7 and 14 days post-immunization.

In striking contrast the lizards held at 25° failed to produce any detectable antibody at 7 days post-immunization and only titers of 1:10 or less after 14 days. Slightly higher titers were obtained in a group immunized at 25° and held at this temperature for one week post-immunization followed by an additional week at 35°C. This latter experiment was repeated in another series (see footnote to Table I) with similar results. Pre-immunization sera were uniformly negative so that this species does not appear to possess a natural agglutinin for *S. typhosa*(H).

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* This investigation was supported in part by grant from Nat. Inst. Health.

TABLE I. Comparison of Antibody Levels in 25 Animals Maintained at 25°C and 35°C.

Temp during immunization	Temp and duration of post-immunization period	Reciprocal of titer
25°C (Group I)*	7 days at 25°C	<10
		"
		"
		"
		"
25°C (Group II)	14 days at 25°C	10
		"
		<10
		10
		"
25°C (Group III)*	7 days at 25°C followed by 7 days at 35°C	20
		10
		20
		40
		"
35°C (Group IV)	7 days at 35°C	80
		160
		"
		640
		160
35°C (Group V)	14 days at 35°C	640
		"
		160
		320
		80

* Group I was repeated by immunizing 10 additional animals exactly as in first experiment. Titers were all below 1:10.

Group III was repeated with 5 additional animals. Titers were, respectively: 40, 80, 80, 80, 20. (Note: in this repetition, the second temperature was 37° rather than 35°C.)

Discussion. These data indicate that antibody synthesis like other metabolic activities in reptiles(4,6,7) is temperature-dependent. At 25°C little or no antibody was synthesized in 7 to 14 days postimmunization, while at 35°C antibody titers of 1:80 to 1:640 were demonstrable after comparatively short period of immunization. It is of course possible that the upper limit for antibody synthesis has not been reached since maximum temperature(4) for *D. dorsalis* is well above 35°C. Further experiments on synthesis and release of antibody in poikilothermic vertebrates are in progress.

Summary. Antibody synthesis at 25°C was compared with that at 35°C in the lizard, *Dipsosaurus dorsalis.* After 2 weeks immunization against *S. typhosa,* H-agglutinin titers of 1:80 to 1:640 were demonstrable in animals maintained at 35°C. At 25°C, on the other hand antibody level did not exceed 1:10 if detectable. Increasing temperature of 25°C group to 35°C for one week caused a relative increase in antibody synthesis. Thus, antibody synthesis in this species appears dependent on environmental temperature.

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Detection of Staphylococcal Antibodies in Human Gamma Globulin and Serum by Hemagglutination Tests.* (24990)

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Plasma fraction II of Cohn, available as commercial gamma globulin, contains many, but not all, antibodies present in blood plasma of random adult population. Its protective value against such virus diseases as measles and infectious hepatitis has been clearly established and that against certain other viral infections is being explored. Recent studies have revealed that human gamma globulin also protects against certain bacterial infections in animals(1-4) and, when used in conjunction with suitable antibiotics, is said to yield favorable results in severe human infections(5,6). Previous investigations suggested that human gamma globulin contains both "complete" and "incomplete" antibodies against somatic (O) antigens of enteric pathogens, including salmonellae, shigellae, and enteropathogenic *Escherichia coli*(7). "Incomplete" antibodies were demonstrated by means of Coombs' hemagglutination and enzyme hemagglutination tests. In the former, antigen modified human erythrocytes were incubated with human gamma globulin and, after washing, tested with human globulin (Coombs') antiserum. In the latter procedure, red blood cells were treated with certain proteolytic enzymes, including trypsin, pancreatic protease, ficin, or papain, either before or after antigen modification, and then tested with gamma globulin. Both tests yielded considerably higher titers than the conventional agglutination and hemagglutination procedures and resemble those used for demonstration of "incomplete" Rh antibodies. Our study revealed that these methods lend themselves also to detection and titration of certain staphylococcal antibodies in human gamma globulin and serum.

Materials and methods. The antigens were prepared as follows. Several freshly isolated strains of *Staphylococcus aureus, citreus,* and *Bacillus subtilis* were grown on brain veal agar in Kolle flasks for 18 hours at 37°C. The resulting growth was removed with 25 ml of phosphate hemagglutination buffer (pH 7.3) (Difco)/flask. The suspensions were centrifuged in refrigerated centrifuge (International, #PR-2, 24,500 G) for 20 minutes and resulting supernates in appropriate dilution employed as antigen. Uninoculated Kolle flasks were treated in like fashion and the washings used for control purposes. Human erythrocytes (blood group O) (2.5% suspension) were washed 3 times with hemagglutination buffer. To the sediment was added the antigen in appropriate dilution in quantities sufficient to restore a 2.5% erythrocyte suspension. The mixtures were incubated in waterbath at 37°C for 30 minutes. The red blood cells were washed 3 times and then used in following tests. *Hemagglutination test*: The antigen modified erythrocytes (vol. 0.2 ml) were added to equal volumes of human gamma globulin or serum in serial dilutions. The mixtures were incubated at 37°C for 30 minutes and centrifuged at 1000 rpm 1 to 2 minutes. The resulting hemagglutination was read grossly. *Enzyme hemagglutination test*: The antigen modified erythrocytes were centrifuged. Pancreatic protease (Nutritional Biochemicals Corp., Cleveland, O.) in concentration of 250 μg/ml was added to the sediment in amounts to yield a 2.5% erythrocyte suspension. The mixtures were incubated at 37°C 30 minutes; the cells were washed 3 times and then tested with gamma globulin or serum as described above. *Coombs' hemagglutination test*: The antigen modified red blood cells were added to gamma globulin or serum in serial dilutions as above; the mixtures incubated at 37°C for 30 minutes; the cells were then washed 3 times. These suspensions (vol. 0.1 ml) were mixed with equal volume of commercially available

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TABLE I. Hemagglutination by Human Gamma Globulin and Serum of Erythrocytes Modified by Staphylococcal Antigen.

Staphylococcal antigens and antibodies		Staphylococcal antigen dilutions (reciprocal)									
		2	10	50	250	1250	2	10	50	250	1250
		Hemagglutination					Protease hemagglutination				
Staphylococcus aureus		Hemagglutinin titers (reciprocal)									
Strain D	(a)*	160	160	160	<20	<20	1280	1280	1280	160	<20
	(b)	80	40	20	<10	<10	80	160	80	<10	<10
Strain C	(a)	<20	<20	<20	<20	<20	1280	1280	160	<20	<20
	(b)	<10	<10	<10	<10	<10	160	160	<10	<10	<10
Strain 6048	(a)	<20	<20	<20	<20	<20	320	20	<20	<20	<20
	(b)	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Staphylococcus citreus											
	(a)	<20	<20	<20	<20	<20	20	<20	<20	<20	<20
	(b)	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

* a = Human gamma globulin. b = Pooled human serum.

Coombs' antiserum, incubated at 37°C 30 minutes, and centrifuged at 1000 rpm for 2 minutes. The resulting hemagglutination was recorded. Several lots of human gamma globulin, including preparations from donors in U.S.A., India (courtesy of Dr. P. R. Dutt, New Delhi), and Japan (courtesy of Dr. I. S. Danielson, Lederle Labs., Pearl River, N. Y.), as well as several pools of inactivated human serum from 20 healthy adults each were employed.

Results. The results of a typical experiment on titers of antibodies in human gamma globulin (U.S.A.) and pooled serum against human red blood cells modified by antigens from 3 strains of *Staphylococcus aureus* and 1 strain of *S. citreus* are summarized in Table I.

Antibody titers in gamma globulin were considerably higher (8 to 128) in the protease hemagglutination than in basic hemagglutination test, when antigens from 3 strains of *Staphylococcus aureus* were used for modification of erythrocytes. It is evident also that the antigen from strain D was active in higher dilution than that from strain C. The antigen from strain 6048 was least active. The degree of increased sensitivity of protease hemagglutination test is related to amount of antigen used for erythrocyte modification and presumably to that present on surface of red blood cells. It is evident that, depending upon source of antigen and amount of antigen used for erythrocyte modification, the protease hemagglutination test yields positive results when the basic test is negative. The Table shows also that protease hemagglutination test is somewhat more sensitive than hemagglutination procedure when human serum is titrated. Repeated experiments yielded essentially similar results. The antigen from *Staphylococcus citreus* was almost completely ineffective. Control experiments, not shown in Table, revealed that sterile fluids prepared from Kolle flasks and used for modification of erythrocytes gave entirely negative results, as did protease treated red blood cells tested with gamma globulin and serum in appropriate dilutions. That merthiolate, used as preservative in commercial gamma globulin, did not contribute to above findings is evident from observations that merthiolate itself in like concentrations failed to agglutinate red blood cells treated with protease and/or staphylococcal antigen, and that merthiolate-free gamma globulin yielded results similar to those obtained with commercial gamma globulin containing this preservative.

Further experiments showed that other coagulase-positive and coagulase-negative strains of staphylococci as well as several strains of *B. subtilis* also yielded antigens suitable for these hemagglutination reactions. Two additional strains of *Staphylococcus citreus* gave negative results. Gamma globulin preparations from India and Japan had antibody titers similar to those present in specimens from U.S.A.

A comparison of Coombs' staphylococcal hemagglutination test with the protease and basic hemagglutination procedures revealed that the former 2 yielded essentially identical titers of antibodies in several lots of commercial human gamma globulin and were considerably more sensitive than the basic hemagglutination method.

Regarding heat stability of staphylococcal antigen, it remained active for erythrocyte modification after heating in boiling water for 15 minutes at pH 2 to 5, but lost its activity when heated at pH 7.2 to 9. In contrast, lipopolysaccharides from gram-negative enterobacteriaceae are more effective in modifying erythrocytes after being heated at alkaline pH(8).

Absorption experiments revealed that suspensions of *Staphylococcus aureus* (strains C and D) and of *B. subtilis* removed the antibody from human gamma globulin, when the latter was tested with red blood cells modified by either of the 3 antigens. In contrast, suspensions of *E. coli* 0111:B4 and 0127:B8 did not remove these antibodies. It is interesting that *Staphylococcus citreus,* which did not yield a suitable antigen for erythrocyte modification, also failed to remove the antibody from gamma globulin.

Discussion. The present investigation revealed that use of red blood cells treated with pancreatic protease and a staphylococcal antigen made possible the detection of antibodies in considerably higher titers in both human gamma globulin and serum than did that of erythrocytes modified by antigen alone. Enhanced sensitivity was noted also when staphylococcal hemagglutination test was combined with the Coombs' method. These observations extend previous studies on basic staphylococcal hemagglutination test used for detection of antibodies in serum(9-13). At least 2 explanations may be considered for results described here. (1) It is conceivable that the enzyme and Coombs' hemagglutination procedures detect "incomplete" staphylococcal antibodies, for such methods are useful for demonstration of "incomplete" Rh antibodies. This possibility, however, must remain speculative, unless it were possible to demonstrate actual differences in antibody molecules between "complete" and "incomplete" staphylococcal antibodies. (2) Alternatively, it is conceivable that the enzyme and Coombs' hemagglutination tests merely render the basic hemagglutination procedure more sensitive and that all 3 methods detect an identical antibody. Since protease and other enzymes(14) are equally effective when used either before or after antigen modification of red blood cells, it is unlikely that they merely make possible the attachment of larger amount of antigen to the surface of cells. Rather, it is suggested that enzymatic removal of some substrate from the surface of red blood cells allows more antigen molecules to participate in the reaction with antibodies and in hemagglutination, as discussed elsewhere(15,16).

It is likely that the staphylococcal antigen is similar to, or identical with, that described by Rantz and associates(17), who demonstrated an antigen common to most gram-positive bacteria and suggested the name "non-species specific (NSS) antigen." Our observations that both *B. subtilis* and *Staphylococcus aureus,* in contrast to *E. coli* and *Staphylococcus citreus,* remove from human gamma globulin the antibodies operative in hemagglutination test, and the fact that the staphylococcal antigen is heat stable at acid, but not at alkaline pH, support this assumption. It is possible also that the antigens common to staphylococcus and streptococcus, described by Pakula and Walczak(10) as well as by Znamirowski *et al.*(11), are of the Rantz type. Further studies are needed to isolate and characterize this antigen. Investigations are in progress to determine the conditions (*e.g.* effect of lysozyme) for antigen release from bacterial cells.

The question arises as to whether the staphylococcal antibodies demonstrated in rather high titers in human gamma globulin by means of the enzyme and Coombs' hemagglutination tests have protective value. Experiments to elucidate this problem are being carried out in collaboration with Dr. Myron Fisher, Detroit, Mich. In particular, the protective effect, if any, of human gamma globulin after absorption with *Staphylococcus aureus, S. citreus, B. subtilis,* and other bacteria is being studied. Should antibodies of the Rantz type be effective *in vivo* in experimental infections, their role in apparently non-specific resistance should be considered along with properdin and other factors.

Summary. Two modifications of a staphylococcal hemagglutination test, utilizing protease for treatment of erythrocytes or Coombs' antiserum, are described and are more sensitive than the basic hemagglutination method for titration of staphylococcal antibodies in human gamma globulin and serum. These antibodies probably are directed against an antigen of the Rantz type, as the antigen is heat stable at acid and labile at alkaline pH and since both *Staphylococcus aureus* and *B. subtilis,* in contrast to *E. coli* and *Staphylococcus citreus,* remove antibodies from human gamma globulin. The significance of the data is discussed.

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New Observations on Pneumococcal Transformations *in vivo*.* (24991)

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The first genetic transformation of pneumococcal types was reported by Griffith(1), who inoculated mice subcutaneously with a mixture of live unencapsulated pneumococci and a vaccine of heat-killed encapsulated pneumococci. The mice developed an infection due to encapsulated pneumococci of the same type as the strain from which the vaccine had been prepared. Since that time most of our information concerning genetic transformation of bacteria has come from studies *in vitro,* although there has been some extension of Griffith's technic *in vivo,* as for example by Austrian(2). We were interested in using purified deoxyribonucleic acid (DNA) preparations for transforming experiments *in vivo.* Previously, only vaccines of intact cells had been used. This report describes results of using partially purified DNA preparations *in vivo,* as well as a modification of Griffith's original technic.

Methods and materials. 1. Strains of *Diplococcus pneumoniae.* SV1, Type I strain virulent.‡ A-66, Type III strain virulent. A-66 1,000, Type III, a mutant obtained from strain A-66 in one step, resistant to 1000 γ/

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‡ Virulence of pneumococci seems to depend directly on amount of polysaccharide capsule(3).

ml of streptomycin. R36NC, an avirulent strain of pneumococcus originally obtained from fully encapsulated Type II D39S strain. It is sensitive to less than 1 γ/ml of streptomycin.

Virulent strains were maintained by weekly passages in mice according to following protocol. One ml of stock culture was transferred to 9.5 ml of fresh blood trypticase soy broth (blood-broth) and incubated 5 to 6 hours at 37°. The culture was diluted to 10^{-4} in trypticase soy broth and 0.5 ml of the diluted solution was injected intraperitoneally into each of 2 mice. The mice were sacrificed 24 hours later and heart blood passed into blood-broth, which was incubated and stored. Blood agar plates were routinely streaked with heart blood of each animal to check for contamination and level of resistance to streptomycin. Swiss female mice, 16-20 g, obtained from Carworth Farms (CF-1) were used. *Vaccine.* The procedure of MacLeod and Kraus(4) was used for growing pneumococcus in quantity. We standardized our preparations by growing each culture until it had taken up 20 ml of 3N sodium hydroxide/liter and then concentrating the cells to 20 ml for each liter of original culture. Vaccines were prepared according to procedure of above authors(4) which essentially consisted of heating fresh concentrate of cells at 65° for one-half hour. All preparations were routinely checked for sterility both *in vitro* and *in vivo*. No vaccines were ever contaminated with live organisms.

Results. 1. *Standard transformation procedure* in vivo. The technic employed was patterned after Griffith's original procedure (1) further modified by MacLeod and Kraus (4). For consistent results it was necessary to use a fresh culture of strain R36NC. One ml of fresh overnight culture of strain R36NC in blood-broth was passed into 10 ml of blood-broth which was then incubated 5 to 6 hours at 37°. The culture was used immediately. One part of culture was mixed with 4 parts of vaccine and 0.5 ml of the mixture was immediately injected subcutaneously into the lower abdominal region of mice. This procedure resulted consistently in nearly 100% mortality of animals that received vaccines prepared from all 3 virulent strains (Table I).

TABLE I. Standard Transforming Procedure (Transfer of Virulence Marker).

Treatment	No. of mice	Total dead 7 days post treatment
SV1 vaccine mixed with R36NC	99	97
" " alone	70	0
A-66 vaccine mixed with R36NC	30	25
" " alone	20	0
A-66-1,000 vaccine mixed with R36NC	20	19
A-66-1,000 vaccine alone	10	0
R36NC culture alone	80	2

Separate groups of mice were inj. (s.c.) with 0.5 ml of a mixture of vaccine and fresh R36NC culture (4:1), 0.4 ml of vaccine and 0.2 ml of fresh R36NC culture.

Lysates of virulent strains. Lysates of all 3 virulent strains were prepared. Physically the results were similar but biologically Type III strain proved most successful. The following procedure used for all 3 strains is described in terms of strain A-66. One hundred ml of concentrated live cells obtained as described above was placed in 125 ml Erlenmeyer flask immersed to its lip in water bath at 65°. The mass was stirred gently with a paddle stirrer for 15 minutes, then 5 ml of fresh 10% sodium deoxycholate solution (aqueous) was added and stirring continued a further 10 minutes while temperature was maintained at 65°. Microscopic examination of the lysate at this point showed extremely few intact cells. The lysate was chilled and 3 volumes of cold 95% ethanol were added with stirring. The precipitate was collected in centrifuge at 2,000 rpm for 20 minutes, blotted with filter paper and resuspended in 100 ml of buffer (0.14 M NaCl, 0.005 M $MgCl_2$, 0.003 M $CaCl_2$, pH 7.1). The fibers were dispersed by forcing the suspension through a No. 24 needle. The suspension was stirred one hour at 0°, then centrifuged for 45 minutes at 27,000 g. The first supernatant was very viscous and opalescent. The procedure was repeated twice more and the residue homogenized in 100 ml of buffer by repeated extrusion through No. 24 needle. This suspension is called A-66 residue. All fractions of the above protocol were analyzed for DNA(5) and carbohydrate(6). The first

TABLE II. Comparison of a Purified Preparation with Vaccine in Standard Transforming Procedure.

Treatment	No. of animals	Total dead 7 days post treatment
A-66 vaccine diluted 20 × with saline mixed with R36NC	10	7
A-66 vaccine alone	5	0
A-66 residue mixed with R36NC	40	39
A-66 residue diluted 20 × mixed with R36NC	10	7
A-66 residue diluted 100 × mixed with R36NC	10	5
A-66 residue alone	40	0
R36NC culture alone	20	0

Separate groups of mice were inj. (s.c.) with 0.5 ml of a mixture of DNA source (diluted or undiluted with physiological saline) and fresh R36NC culture (4:1), 0.4 ml undiluted DNA source alone, and 0.2 ml of fresh R36NC culture alone.

homogenate, the ethanol precipitate in the buffer solution contained 2500 γ/ml of DNA and 1700 γ/ml of carbohydrate. The resuspended A-66 residue contained 2200 γ/ml of DNA and 660 γ/ml of carbohydrate. Practically all the sugar was removed in the first wash. The A-66 residue had as much transforming activity as a comparable volume of heat-killed vaccine prepared from the A-66 strain (Table II). It could be stored at 4° for at least 2 months without losing transforming activity. The results were very reproducible. Six separate runs gave same results each time. The infecting organisms resulting from transforming experiments in which the A-66 residue was used were isolated as follows. Twenty mice received a single subcutaneous injection of a mixture of 4 parts A-66 residue and one part of a culture of strain R36NC. Forty-two hours later all mice were very sick. Ten were sacrificed. The remaining mice died within 2 days. Heart blood of each sacrificed animal was passed into blood-broth tubes. After overnight incubation 8 of 10 tubes contained a bacterial growth, which in each case was due to a Type III pneumococcus. Virulence of the 8 cultures was established by injecting intraperitoneally 0.5 ml of a 10^{-6} dilution of fresh cultures into mice (10 mice for each culture). Within 48 hours, 70 of the 80 mice died. Four of the remaining mice (all sick) were sacrificed and the infecting organism was isolated. It was in each case a Type III pneumococcus.

The residue prepared from the SV1 strain according to above procedure gave similar chemical analyses indicating that most of the original DNA content of the cells was still in the residue. But at best, this residue had weak transient activity in the standard transforming system. Results were inconsistent over several runs; in general this residue was inactive.

Inactivation of A-66 residue with detergent or chloroform. Twenty ml of the A-66 residue was shaken vigorously with 20 ml of chloroform and 0.6 ml of *iso*amyl alcohol for 5 minutes. The aqueous layer was immediately assayed for transforming activity in the *in vivo* system and found to be inactive. Twenty ml of A-66 residue was stirred 3 hours at 0° in presence of 2 ml of 5% dodecyl sulfate solution. The solution was centrifuged at 27,000 g. for 20 minutes, after which 3 volumes of ethanol were added to the clear supernatant. The fibrous precipitate was immediately homogenized in 20 ml of normal saline and assayed in the standard transforming system. It was completely inactive.

Transformation of streptomycin sensitive pneumococci into resistant pneumococci. Twenty mice were injected subcutaneously with 0.5 ml of a 1:4 mixture of a fresh culture of R36NC strain and residue from strain A-66-1,000 obtained in same manner as A-66 residue. Seven hours later, 10 mice were injected subcutaneously with 400 mg/kg of streptomycin sulfate (data in Table III show that a 7 hour post-infection administration will protect about 80% of mice infected with the SV1 strain). Nine of the 10 mice not re-

TABLE III. Protection by Streptomycin, of Mice Infected with SV1 Strain of Pneumococcus.

Hr post infection single dose (s.c.) of 400 mg/kg of streptomycin sulfate	Total mortality 6 days post infection
0	0/10
7	2/10
16	9/10
24	10/10
40	10/10
No streptomycin	10/10

Mice were infected with 0.5 ml of a 10^{-6} dilution of a fresh 6 hr culture of SV1 pneumococcus.

ceiving streptomycin died within 3 days post infection. The 10 mice which received streptomycin were sacrificed 42 hours post infection. Heart blood from each animal was streaked on blood agar plates. After 24 hours incubation, the 10 plates were covered with smooth glistening colonies. The plates were replicated(7) to blood agar plates containing 0, 5 and 1,000 γ/ml of streptomycin sulfate. All 10 isolates showed equal number of colonies on all 3 replicates. A similar experiment starting with an A-66 vaccine resulted in no colonies that would grow on blood agar plates containing 20 γ/ml of streptomycin sulfate.

Variation on Standard Transformation Technic in vivo. Vaccines from strains SV1, A-66, and A-66-1,000 or the A-66 residue were injected subcutaneously *per se* and the avirulent strain R36NC was injected intraperitoneally *per se*. This modification was about 70% as effective as the classical procedure judging from mortality of animals (Table IV). The R36NC culture did not have to be injected simultaneously with the vaccine injection; indeed, successful transformation still occurred when injection of the R36NC culture was delayed 24 hours after vaccine injection (Table V).

Discussion. Many workers have used Griffith's technic for transformation of avirulent pneumococci to virulent pneumococci *in vivo*. We used his technic essentially as a control for biological activity of the vaccine and lysate preparations. By paying close attention to the condition of the avirulent culture (R36NC), nearly 100% mortality of treated groups was consistently obtained. The results also showed that amount of vaccine necessary for successful transformation could be reduced at least 50-fold from that used by Griffith.

TABLE IV. Modified Transforming Procedure *In Vivo*.

Treatment	No. of mice	Total dead 7 days post treatment
SV1 vaccine + R36NC	54	35
SV1 vaccine	10	0
A-66 " + R36NC	10	6
" residue + "	10	6
" vaccine	10	0
" residue	10	0
R36NC	10	0

Mice received 0.5 ml of vaccine or residue subcut. and 0.2 ml of fresh R36NC culture intraper. Control groups received only a single inj. of either the transforming factors or R36NC culture in the same amount.

TABLE V. Transfer of Virulence Marker *In Vivo* by Modified Transforming Procedure.

Hr between administration of SV1 vaccine (s.c.) and R36NC culture (i.p.)	Total dead 5 days post treatment
0	8/10
4	8/10
8	10/10
24	10/10
Vaccine alone	0/5
R36NC alone	0/5

Separate groups of mice were inj. (s.c.) with 0.5 ml of 1:1 dilution of SV1 vaccine prior to inj. (i.p.) of all groups at the same time with 0.2 ml of a fresh 6 hr culture of R36NC.

Injection of an animal with a mixture of transforming material (vaccine) and an avirulent culture subcutaneously, results in a pocket where intimate contact between components of mixture is maintained for considerable time before complete absorption by host. Transformation could occur during this time which could hardly mean true involvement of host in the process. This objection was met by injecting the transforming material and live avirulent culture separately at different sites in the animal. The positive results of this technic mean that interaction of the 2 components of the transforming system was mediated by circulatory action within the living host. Transformation of pneumococcal capsule types *via* this technic, although not as efficient as *via* the standard procedure was achieved with consistent results with several batches of vaccines and DNA lysate preparations. The technic of separate injections affords a method for studying survival of biologically active DNA in the living animal. In one experiment vaccine prepared from the SV1 (Type I) strain was injected 24 hours prior to injection of the live avirulent cells. The treated animals died indicating that DNA with transforming activity in this system survived *in vivo*, at least 24 hours.

One of the main objectives of this program was to make purified DNA transforming preparations that would be active *in vivo*. Certainly, for *in vitro* transforming systems, highly purified DNA samples can be successfully used. Austrian tried a purified, relatively protein-free DNA preparation, that would have had transforming activity *in vitro*; but it was inactive *in vivo*. First steps toward an active *in vivo* preparation were taken by heating fresh live cells in presence of deoxycholate, similar to procedure used by Alloway for DNA preparation used *in vitro*(8). This procedure essentially accomplished 3 things: lysis, sterility, and freedom from nucleases. The lysate was diluted with ethanol and the resultant precipitate washed twice with dilute buffer. Practically all the original DNA remained in the final residue. Physical and chemical response of the 2 strains, SV1 and A-66, to this procedure was similar. Only the residue from the Type III strain (A-66) was active in the *in vivo* transforming system. In fact this preparation was as active as a comparable amount of vaccine prepared from same strain. It could be stored in aqueous suspension at 4° for several weeks without impairment of activity. There was no apparent reason for lack of parallel activity in the Type I preparation.

The partially purified transforming principle from the Type III strain was inactivated by mild protein denaturation caused by either the chloroform procedure or detergent procedure. It is well known that such procedures are not deleterious to *in vitro* transforming activity of DNA preparations(9,10). The loss of transforming activity *in vivo* caused by these procedures might infer that the protein conjugate is required for stability of DNA in an *in vivo* system; the suggestion may be that the physical structure of DNA must be more intact for biological activity in an *in vivo* system than in an *in vitro* system.

Streptomycin resistance can be conferred on a streptomycin sensitive pneumococcus *via* a transforming preparation *in vivo*. Actually, the experimental results described here depended on double transformation of both virulence and streptomycin resistance markers to the recipient R36NC culture. The transformed bacteria were resistant to 1000 γ/ml of streptomycin sulfate, the resistance of donor culture, which was a "one-step" mutant of strain A-66. Langvad-Nielsen performed a similar experiment but omitted the streptomycin injection with the result that only the virulence marker was transferred(11). Austrian has reported a double transformation *in vivo* of type specific capsular polysaccharide and type specific M protein(2).

Summary. 1. Succesful pneumococcal transformation *in vivo* has been achieved by injecting the transforming preparation subcutaneously and the live avirulent culture intraperitoneally. 2. The transforming activity of a vaccine preparation lasted *in vivo* at least 24 hours. 3. Transforming preparations active *in vivo* have been prepared from a lysate of a culture of strain A-66 (Type III). This preparation was inactivated by mild protein denaturation. 4. The genetic marker of streptomycin resistance was transferred between pneumococcal strains *in vivo*.

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Thoracic Duct Lymph in Unanesthetized Mouse. Method of Collection, Rate of Flow and Cell Content.* (24992)

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A method has been described for short term collection of thoracic duct lymph from the anesthetized mouse(1). The following report is concerned with the possible difference between lymph flow and lymphocyte output in unanesthetized as contrasted with anesthetized state. In addition, thoracic duct lymph flow and lymphocyte output collected from abdominal or cervical regions of anesthetized mice are compared.

Technic. Male mice of NIH-Webster strain, having mean body weight of 24 g ± 1, maintained on Purina Laboratory Chow, were used. Operative procedures were carried out under sodium pentobarbital-induced anesthesia (7 mg/100 g B.W., injected intraperitoneally). With this dose level, the animals recovered consciousness and became active within approximately one hour. The abdominal thoracic duct was cannulated caudal to diaphragm with polyethylene tubing (internal diameter 0.011 in.), utilizing with minor variations a method employed for rats(2). For instance, a small thin-edged spatula was used to separate thoracic duct from aorta. A curved atraumatic needle(6-0), the thread of which was looped, was used to pull a second silk thread (50 gauge) around the thoracic duct to fasten the cannula in place; a second restraining tie was placed around the cannula in the duct and fixed to body wall to prevent dislodgment. After operation a thin glass rod was attached in the sagittal plane to the skin on dorsum of animal by means of wound clips. This rod partially restrained the mouse from forward, backward, or lateral movements. The mouse was placed by means of glass rod on upper and outer circumference of an activity wheel (Fig. 1). This arrangement permitted voluntary rest, or freedom to exercise, as well as access to fluids and food. After recovery from the anesthetized state, animals were active, ate food, and drank water during period of lymph collection, and appeared healthy at end of collection period. Lymph was collected at room temperature into a glass vial which contained a small quantity of sequestrene (ethylene diamine tetraacetate) to prevent coagulation. Lymph flow and lymphocyte output were measured for successive 6-hour samples over 24 hours from each of 17 mice. A few animals were permitted to drain lymph for 30 hrs; one animal continued lymph flow for 48 hrs. Duplicate white blood cell counts were carried out on each 6-hour sample of lymph. Occasional clotting of lymph, usually in the first 6 hrs., and at the distal end of cannula, was relieved by slight negative pressure at cannula tip. Comparative studies

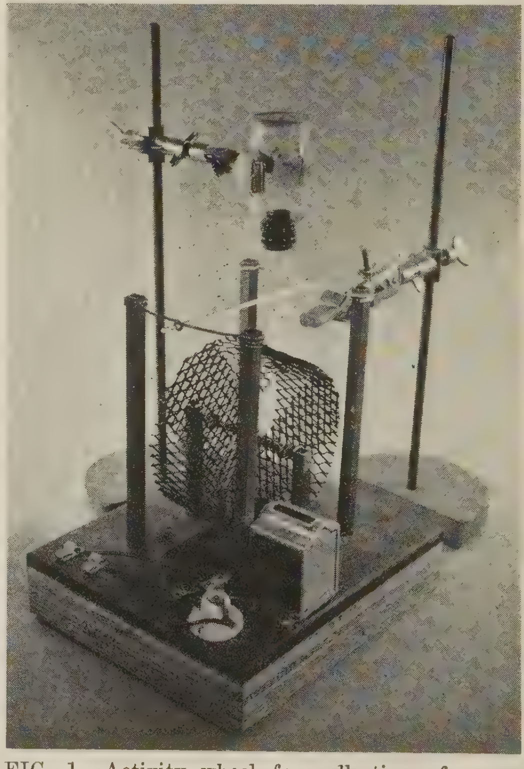

FIG. 1. Activity wheel for collection of mouse thoracic duct lymph. Water bottle, food, and adjustable restraining glass rod are shown.

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TABLE I. Effect of Anesthesia and of Site of Collection on Thoracic Duct Lymph Flow and Cell Content in Male Mice, in 2 Experiments.

Treatment	No. of animals	Collection time, hr	Lymph vol, ml/hr	Lymphocyte Content cells/mm³ (× 10³)	Lymphocyte Output cells/hr (× 10⁶)
Abdominal thoracic duct					
Unanesthetized	17	6	.06 ± .01*	30.8 ± 2.1	1.5 ± .2
Anesthetized†	10	3	.07 ± "	27.5 ± 4.4	1.4 ± .1
Anesthetized†					
Abdominal thoracic duct	7	1	.09 ± .01	20.0 ± 2.7	1.6 ± .2
Cervical " "	7	1	.08 ± "	21.0 ± 4.2	1.6 ± .3

* Mean ± stand. error. † Intraper. inj. of solution of sodium pentobarbital.

were made on 10 anesthetized mice by collecting abdominal thoracic duct lymph for 3 hours. Seven pairs of male mice were used in comparative studies of thoracic duct lymph flow and cell output in anesthetized animals, lymph being collected from the abdominal thoracic duct in one of each pair and from the neck in the other, using a technic for the latter as described for the rat(3). Because of previously demonstrated diurnal variation in the mouse(1), lymph collections were carried out at similar times for each pair of animals. Significance of difference of means was calculated, using Fisher's values for t, p values of $<.01$ being considered of probable significance.

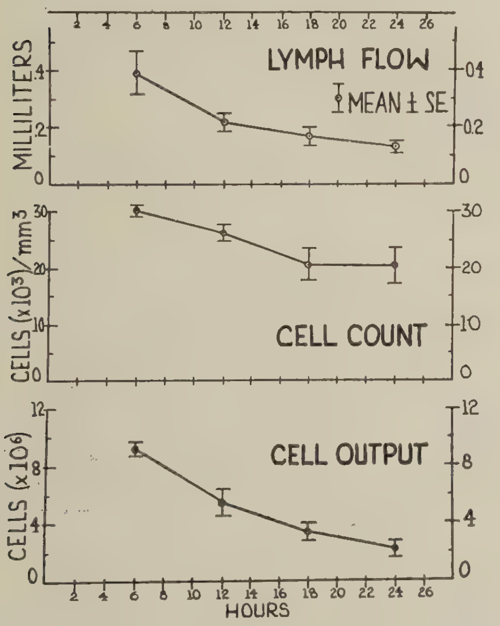

FIG. 2. Changes in lymph flow, lymphocyte count and total lymphocyte output, at 6 hr intervals, in mice with continuously draining abdominal thoracic duct lymph.

Results. Fig. 2 presents values, for each of 4 consecutive 6-hour periods, for lymph flow, lymphocyte content, and total lymphocyte output, for 17 male mice. Lymph flow decreased sharply after first 6 hours, then declined slowly over remaining collection period. Lymphocyte count decreased about one-third in the first 18-hour period, then leveled off. Total lymphocyte output decreased at every 6-hour interval. Comparison of first and second 6-hour samples revealed a significant decrease in lymphocyte output. The fall in cell output was further extended in animals maintained for 30 hours and in the animal allowed to drain lymph for 48 hours. In the latter animals, however, lymph volumes did not vary from those obtained at end of first 24 hours collection. These findings confirm the continuous fall in thoracic duct cell output reported for unanesthetized rats(4,5,6).

No consistent relationship was noted between lymph flow and lymphocyte output in either unanesthetized or anesthetized animals (Table I). Data derived from lymph studies in the unanesthetized state are therefore deemed to be representative of values obtained in anesthetized mice only for a limited period after recovery from the anesthetized state. Similar results have been recorded for rats(7).

Technics of collection of thoracic duct lymph from abdominal and cervical regions were assessed. Both technics are of equal difficulty and for short-term studies either technic is adequate. However, for longer drainage of lymph from anesthetized forms, the abdominal approach is preferred. The cervical approach was generally accompanied by respiratory distress after more than one hour of lymph collection.

Table I also presents a comparison of data obtained from studies made on paired mice in which thoracic duct lymph was collected from either the neck or abdomen at same time of day. Mean values for lymph flow, lymphocyte content, and total lymphocyte output did not differ. Although mean values were similar for abdominal and for cervical lymphocyte output for paired animals, individual cell outputs collected simultaneously in paired animals on same day were closer to each other than in collections made at similar times but on different days.

On the basis of the mean total thoracic duct lymphocyte output of 1.5 cells/hr x 10^6 for anesthetized or unanesthetized mice, it can be calculated that at least 35 million cells enter the blood from this duct in a 24-hour period. This is 3.3 times the total calculated number of circulating lymphocytes.

Summary. 1. The mouse is a suitable animal for collection of thoracic duct lymph in the partially-restrained unanesthetized state. 2. Initial values for lymph flow and lymphocyte output did not vary significantly in the unanesthetized as compared to the anesthetized mouse. 3. A continuous decrease in lymph flow and lymphocyte output from the thoracic duct occurred over a 24-hour period in the unanesthetized mouse. 4. Values for thoracic duct lymph flow and lymphocyte output did not vary significantly, whether collected from the neck or abdomen.

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Cytopathology of Feline Viral Rhinotracheitis Virus in Tissue Cultures of Feline Renal Cells. (24993)

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In the original report(1) on isolation of a feline virus associated with intranuclear inclusion bodies the agent was designated as C-27 virus. We now propose the name "feline viral rhinotracheitis" for the disease entity induced by this agent. The virus in this and future communications will be referred to as feline viral rhinotracheitis (FVR) virus. In this paper we describe the sequence of morphological changes with formation of multinucleated giant cells or syncytia in cell cultures infected with FVR virus, and report our results from the use of different methods of fixation for demonstration of inclusions.

Materials and methods. The original FVR virus obtained from the throat of a cat exhibiting clinical signs of upper respiratory infection was isolated in cultures of feline renal cells. The virus used was from 5th and 6th tissue culture passages. Primary cultures of feline renal cells were prepared on 11 x 22 mm coverslips in Leighton tubes. Tissue culture methods employed were those of Madin *et al.* (2). The cells were grown in nutrient medium consisting of 0.5% lactalbumin hydrolysate in modified Hank's salt solution containing 0.01 M Tris (Hydroxymethyl aminomethane), adjusted to pH of 7.4, and 10% lamb serum. The maintenance fluid used at time of inoculation was lactalbumin hydrolysate

nucleoli fragment into fine particles dispersed on light-staining background of nuclear substrate (Fig. 2). These granules then marginate, leaving a central area of homogeneous nuclear material (Fig. 3). Later this nuclear material becomes acidophilic and contracts, forming a well-developed inclusion surrounded by clear halo (Figs. 4 and 5). Outside the halo the basophilic nuclear membrane remains intact though often somewhat shrunken. In a few cells which retain their nucleoli, 2 or more acidophilic intranuclear inclusions with small individual halos have been observed (Fig. 6).

Characteristic cytoplasmic change is formation of multinucleated giant cells or syncytia. Individual cell loses its distinct cellular membrane and fuses with adjacent cells. In early stages the cytoplasm of cells in the syncytia is paler than in other cells (Fig. 7). These syncytia contract and eventually break away from the cellular mass to form separate multinucleated giant cells (Fig. 8). In the final stage of development, these giant cells are round or club shaped and have a granular, intense eosinophilic cytoplasm with nuclei about the periphery (Figs. 9 and 10). In these nuclei are inclusions at various stages of development. No specific cytoplasmic inclusions were found.

Discussion. Although intranuclear inclusions were demonstrated at the time this virus was first isolated, the significance of syncytium formation was not initially recognized as a feature of its cytopathogenicity. This belatedly recognized cytopathic change was suggestive of a possible mutation of our original isolate or the introduction of another agent. Upon reexamination of H & E preparations of our earlier passages, we observed occasional small giant cells with 2 or more nucleoli. Only one type of plaque was produced in cultures by the method described by Dulbecco and Vogt(3). All of these cytopathogenic changes, now considered characteristic of FVR virus, were inhibited by homologous immune rabbit serum.

It appeared unlikely that the tendency to formation of giant cells was a nonspecific alteration in the feline cultures, since primary cultures had been used in all our studies. In addition, we used cultures of feline renal cells for several years and never previously experienced this type of cytopathogenic change in uninfected cultures. Development of typical intranuclear inclusions is observed in these giant cells. In later tissue culture passages, these multinucleated giant cells are larger and occur with greater frequency. We observed nuclear changes and well-developed inclusion bodies in necropsy material from cats experimentally infected with the virus. However, giant cells similar to those found in tissue cultures have not been observed in the disease.

In this work, metabolism of cultures was measured by acid production in the media. Comparison of hydrogen ion concentration of fluids from noninfected and infected cultures indicate that there is a slight metabolic inhibition in the infected cells. Reduction in pH of fluids is less for infected cultures than for normal. Although there is a pH differential between fluids of infected and noninfected cultures, it is not sufficient to apply the color test for infectivity in the tissue culture system studied. Robbins *et al.*(4) reported that metabolic activity of cultures infected with polio virus was less than that of normal control cultures. In tissue cultures infected with adenovirus(5) and the virus of infectious bovine rhinotracheitis(6), the reverse is seen. With these latter viruses, infection results in a decrease in pH of culture fluids. Recognition of these basic differences characteristic of these viruses leads to a better understanding of the virus-cell relationship.

Of fixatives studied, inclusion bodies were demonstrated in H & E preparations which were fixed in Bouin's fluid, Zenker's acetic, methanol, and acetone. Bouin's fixation consistently gave the best results and is used routinely in our laboratory. Formalin-fixed tissue culture preparations were unsatisfactory for studying the cytopathology, and inclusion bodies were not demonstrable when such preparations were used.

The virus was propagated in primary cultures of feline lung and testicle, resulting in development of cytopathogenic changes and inclusions similar to those occurring in cultures of feline renal cells. Cytopathogenic changes were not demonstrated in cultures of bovine kidney, Chang liver, human amnion, monkey kidney, human conjunctiva, and "L" cells.

Summary. Cytological changes occurring in cultures of feline renal cells infected with virus of feline viral rhinotracheitis are described. The features of cytopathogenicity of this virus are (1) development of intranuclear inclusion bodies; (2) formation of giant cells; and (3) a slight reduction in metabolic rate of infected cells.

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Effect of Midbrain Lesions on Ovulation and Adrenal Response to Stress in Female Rats.* (24994)

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As part of a continuing study attempting to identify brain areas essential for normal pituitary function effects of localized brain lesions on stress-induced corticosterone release and adrenal ascorbic acid decrease have been investigated. Earlier experiments revealed a dichotomy between adrenal ascorbic acid decrease and corticosterone release following surgical stress in rats with electrolytic lesions placed in different parts of the hypothalamus(1). In the present study adrenal responsiveness has been measured in rats with lesions in the diencephalon, midbrain and at the level of rostral pons. In addition, since lesions in the midbrain have been shown to inhibit ovulation(2) changes in ovulatory cycle were investigated in some animals.

Materials and methods. Coagulating brain lesions were made in 28 adult female Sprague-Dawley rats (225-300 g) from our breeding colony. The coagulating current (2.7 millicycles/sec.) was delivered for 5-10 sec. from a Birtcher Blendtome surgical unit through paired stainless steel insect pin electrodes 1 mm apart, insulated except for 1 mm at tip. Midline lesions were made at various levels between posterior border of mammillary body and posterior end of the interpeduncular nucleus 1 to 3 mm from base of brain. In control rats electrodes were inserted but no current applied. Rats were maintained postoperatively in individual cages on Purina lab chow or force-fed 3-5 times daily with milk-5% glucose solution. Constant room temperature (37-38°C) and controlled lighting conditions were maintained(2). Four days after lesioning, the left adrenal vein was cannulated and blood collected for 30 minutes. Pre- and post-stress adrenal ascorbic acid levels were determined immediately after cannulation ("resting" levels) and at end of collection(3), using the method of Roe and Kuether(4). Corticosterone was analyzed as noted earlier(5). When necessary, anesthesia was induced by pentobarbital sodium (3 mg/100 g, i.p.). Nine rats were studied for changes in ovulatory cycles by method described by Critchlow(2). In these rats, unilateral ovariectomy was performed on first or second day post-lesioning. No rat was included in the series if microscopic examination of pituitary revealed damage from lesioning.

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TABLE I. Effect of Lesions on Adrenal Ascorbic Acid Decrease, Corticosterone Release and Ovulation in Female Rats.

			Adrenal	
			Ascorbic acid	
Group	No. rats	Corticosterone, total μg	Resting level, mg %	% change
A Controls: no lesion	7	33 ± 2*	424 ± 28	−24 ± 2
B Midbrain lesion: central area between lesion Groups C and D	7	40 ± 1	431 ± 26	−27 ± 3
	2	*37*† *(43, 30)*	*325* *(313, 336)*	*−6* *(0, −12)*
C Lesion: level of posterior midbrain and rostral pons	3	55 (52, 54, 58)	461 (427, 438, 518)	−23 (−21, −22, −26)
	4	*68* *(56, 61, 61, 92)*	*302* *(281, 288, 317, 320)*	*−18* *(−7, −10, −17, −37)*
D Lesion: posterior diencephalon, rostral midbrain	2	19 (19, 19)	387 (369, 405)	−15 (−15, −15)
	3	*18* *(15, 20, 20)*	*376* *(352, 381, 394)*	*−6* *(0, −5, −12)*

* Mean ± S.E. of mean. † Italicized data: ovulation blocked; unilateral ovariectomy.

Animals were divided into 3 groups on the basis of location of brain lesion and corticosterone secretion rates (Table I).

Results. Lesioned areas. The central core of midbrain was extensively damaged in all lesioned rats. The lesioned area common to rats exhibiting depressed corticosteroid levels (Table I, Group D) involved posterior diencephalon (subthalamus and dorsal hypothalamus) and extended into rostral midbrain (Fig. 1). In contrast, the lesioned area common to rats exhibiting high corticosterone levels (Group C) involved posterior midbrain and extended into the level of rostral pons. In the animal of this group exhibiting highest release of corticosterone (92 μg), the most extensive lesion was observed. Only fibers of the lateral lemniscus connected anterior and posterior portions of the central nervous system. Midbrain lesions (Group B) not involving either the area of posterior diencephalon damaged in Group D animals or posterior midbrain and rostral pons level destroyed in Group C animals did not alter corticosterone levels. Brains of rats in which ovulation was shown to be blocked were damaged in region of mammillary peduncle as described previously by Critchlow(2). However, this area did not appear to exert any control on corticosterone secretion since it was undamaged in some rats of each group.

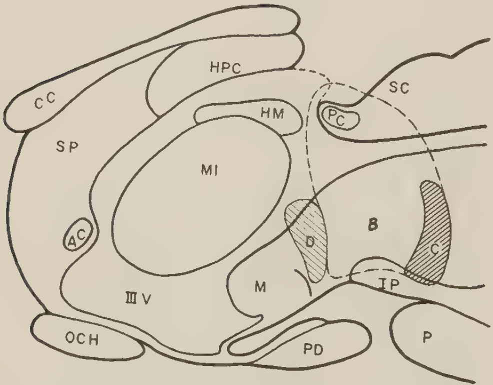

FIG. 1. Sagittal reconstruction of rat brain showing lesioned area common to all rats exhibiting inhibition of adrenal ascorbic acid and corticosterone response to stress (D), area resulting in increased corticosterone release (C) and area resulting in normal corticosterone release (B). Dashed outline representative of largest lesion evoking increased corticosterone release. Abbreviations: AC, anterior commissure; CC, corpus callosum; HM, N. habenularis medialis; HPC, hippocampal commissure; IP, interpeduncular nucleus; M, mammillary body; MI, massa intermedia; OCH, optic chiasm; P, pons; PC, posterior commissure; PD, pars distalis; SC, superior colliculus; SP, septum pellucidum; III V, third ventricle.

Corticosterone levels. The left adrenal gland of 7 control rats released an average of 33 μg corticosterone into adrenal venous effluent during the first 30 minutes post-cannulation (Table I, Group A). Comparable amounts were released in 9 lesioned rats (Group B). In contrast, 7 lesioned rats exhibited significantly elevated levels of corticosterone (Group C),—in one instance 3 times (*i.e.* 92 μg) that noted in controls. Significantly depressed levels of corticosterone were recovered from 5 lesioned rats (Group D).

Adrenal ascorbic acid (AAA). AAA response varied from normal to complete inhibition in groups B and C and was uniformly low in Group D (Table I). Resting levels of AAA were generally depressed in the unilateral ovariectomized rats in each of the lesioned groups. These animals in most cases exhibited a lesser AAA response to stress than unovariectomized members of the same group. These results in ovariectomized animals were probably due to additional stress of this operation.

Ovulatory response. In 9 of the lesioned rats the effect of midbrain damage on ovulation was checked; in each instance ovulation was observed to be inhibited. The inhibition of ovulation appeared independent of either adrenal response (Table I).

Conclusions. Since gross lesions involving posterior portion of midbrain and area at level of rostral pons resulted in increased corticosterone recovery it would appear that these structures may normally exert an inhibitory effect on adrenocorticosteroid response to stress. These findings are consistent with observation of Newman, *et al.*(6) that cortisol secretion was increased in 3 of 4 cats with brain stem lesions involving rostral pons. Other areas previously implicated in possible neural inhibition of adrenal cortical function include rhinencephalon(7) and, specifically, amygdala(8). In contrast, lesions of posterior diencephalon and rostral midbrain resulted in depressed corticosterone levels following stress, similar to those found earlier after dorsal posterior hypothalamic lesions(1). These data suggest that certain structures in posterior diencephalon and rostral midbrain must be intact for corticosteroid response to stress. The present studies confirm the previous finding that AAA and corticosterone responses to stress may vary independently following brain lesioning(1). The same brain stem areas do not appear to be involved in control of the 2 indices of adrenal function.

In addition, the data presented indicate that the midbrain area influencing the ovulatory cycle is distinct from centers concerned with ACTH regulation.

Summary. Coagulating lesions in posterior portion of midbrain and level of rostral pons of female rats resulted in increased corticosterone secretion into adrenal venous effluent in response to surgical stress. Lesions in posterior diencephalon and rostral midbrain structures resulted in depressed corticosterone secretion. Adrenal ascorbic acid response to stress was not correlated with corticosterone response. Ovulation was blocked in rats with brain lesions in any of these areas where concomitant mammillary peduncle damage was present.

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Effect of Insulin and Cortisol on Organ Cultures of Adult Mouse Mammary Gland.* (24995)

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Effects of hormones on organ cultures of adult mouse mammary gland are being investigated in this laboratory. Previous reports (1,2) concerned responses of mammary explants after addition of various ovarian, adrenal, and pituitary hormones to a defined medium. This report describes the effects of adding insulin and cortisol.

Materials and methods. The experimental procedures used herein have been described (1,2). All tissues were derived from "high mammary tumor" (agent-bearing) C3H/He Crgl female mice. Prelactating explants were taken from mammary glands between 14th and 18th days of pregnancy (Fig. 1). Multiparous females, both with and without spontaneous mammary tumors, provided hyperplastic alveolar mammary nodules(3) for cultivation. Most explants were about 1 x 1 x 0.5 mm in size. The tissues were cultured for 5 days at 37°C, using floating lens paper method of organ culture(4). After cultivation, explants were fixed in Bouin's fluid, and serial sections of paraffin-embedded material stained with modified Masson's trichrome stain (containing iron hematoxylin, acid fuchsin, Biebrich scarlet, and aniline blue). The defined culture medium was "199"(5) supplemented with cortisol and insulin, alone and in combination. The crystalline Zn-insulin preparation used (Eli Lilly #535664) had 25 units of activity/mg. It was dissolved in "199" to produce a concentration of 140 μg/ml in final medium. The non-esterified cortisol preparation was first dissolved in 100% ethanol, then added to "199" to provide a concentration of 8 μg/ml of cortisol and 0.5% ethanol in final medium. *Normal histology.* The prelactating mammary gland from 14th to 18th day of pregnancy consisted of well developed lobules of alveoli showing histologic evidence of secretory activity (Fig. 1). The alveolar cells possessed large intracellular vacuoles, and lumina of moderately enlarged alveoli contained secretory material composed of a stainable granular material and extruded vacuolar contents (lipids). Hyperplastic alveolar nodules, in general, had an histologic appearance similar to that of prelactating lobules(6), but occurred in otherwise inactive glands.

Results. A total of 72 prelactating explants derived from 12 animals, and 26 hyperplastic nodules from 10 animals, were used. Since prelactating lobules and hyperplastic alveolar nodules responded similarly, the results are presented together. Tissues cultured in "199" alone, or in "199" containing added cortisol, underwent extensive alveolar degeneration after 5-day culture period (Fig. 2). Addition to "199" of a combination of cortisol + insulin resulted in alveolar maintenance in all explants during the 5-day culture period. The cuboidal alveolar cells appeared healthy, but contained no intracellular vacuoles. Alveoli were generally distended (Fig. 3), often markedly so (Fig. 4). The lumina of most alveoli showed little or no stainable material, but a few contained some stainable secretion. The material in the lumina of most of the distended alveoli was not sudanophilic.

Addition of insulin alone to the "199" also improved survival of explants (Fig. 5), but not so well as those cultured with both cortisol and insulin. On the other hand, Trowell(7) apparently obtained good maintenance of rat mammary explants using his synthetic medium (containing insulin) and a high oxygen gas phase. He further reports "progressive reduction in secretion content of cells," but details are not given, and the accompanying Figure shows a highly vacuolated alveolar epithelium.

Appearance of alveoli following cultivation in cortisol + insulin-supplemented medium

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* Aided by research grants from Am. Cancer Soc.

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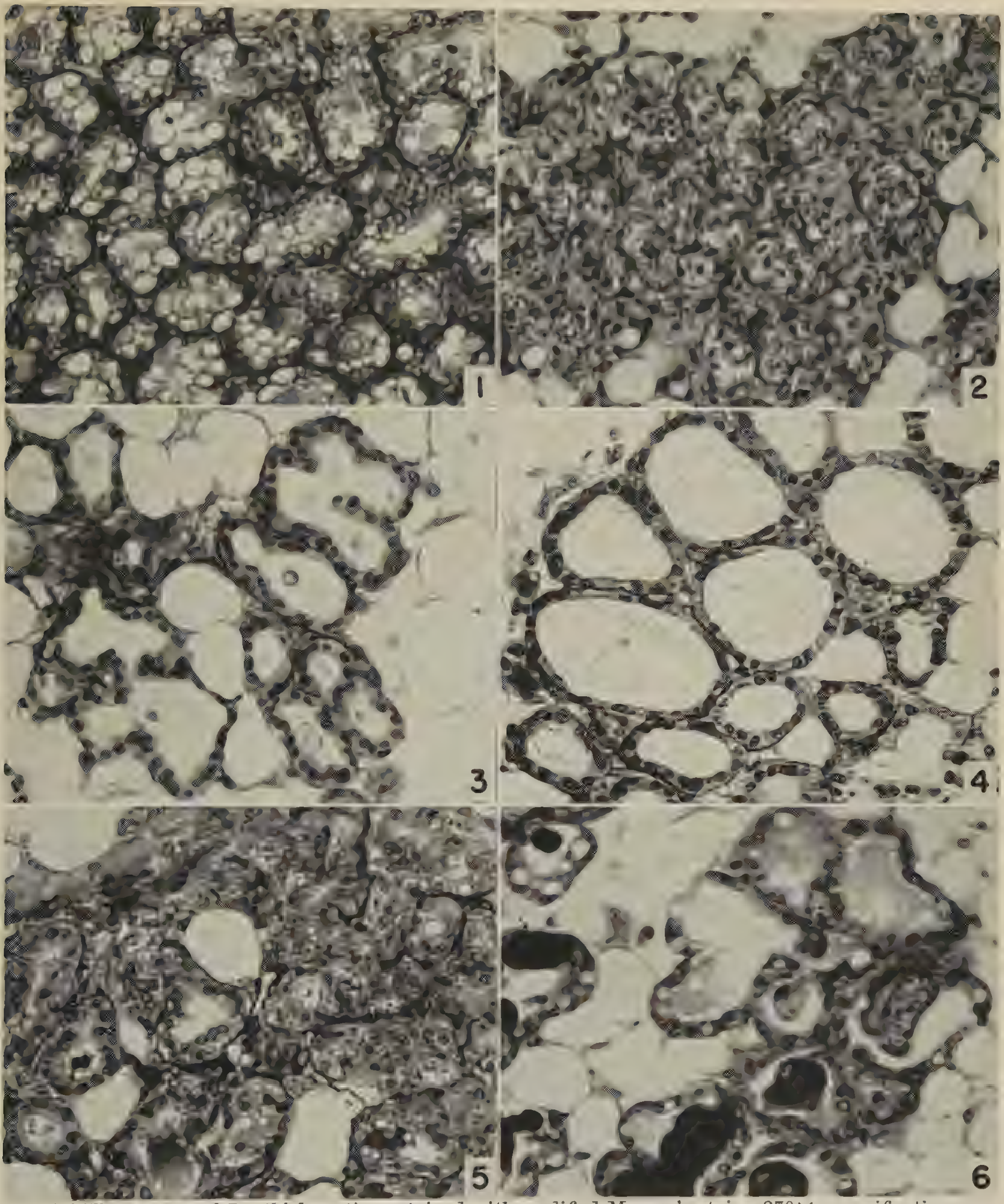

All Figures are of 7 μ thick sections stained with modified Masson's stain. 270× magnification.

FIG. 1. Normal prelactating mammary gland of C3H/He Crgl mouse at about 15-16th day of pregnancy. Note large intracellular vacuoles, secretory material in lumina of alveoli.

FIG. 2. prelactating explant after 5 days cultivation in "199" with added cortisol, 8 μg/ml, showing degeneration of alveoli.

FIG. 3. Prelactating explant after 5 days cultivation in "199" with added cortisol + insulin, 8 + 140 μg/ml. Note loss of intracellular vacuoles, distension of alveoli, unstained contents of alveolar lumina.

FIG. 4. Prelactating explant, treated as in Fig. 3. Note extreme distension of alveoli.

FIG. 5. Hyperplastic nodule explant after 5 days cultivation in "199" with added insulin, 140 μg/ml. Note loss of alveolar organization in area in upper right part of Figure. Some alveoli have *extra*cellular vacuoles and stainable material in their lumina.

FIG. 6. Prelactating explant after 5 days cultivation in "199" with added cortisol + mammotropin, 8 + 140 μg/ml. Compare to Fig. 3 and 4.

differs from that seen after addition of cortisol + mammotropin to the culture medium (1,2). Cortisol + mammotropin-treated alveoli contain many intracellular vacuoles and a dense, darkly-stained secretion in alveolar lumina (Fig. 6), in contrast to loss of intracellular vacuoles and the unstained nature of luminal contents following cortisol + insulin treatment. Thus, there is more than one hormonal combination that will maintain prelactating and nodular alveoli in culture, but there are differences in the resulting histologic picture.

Summary. Cortisol plus insulin, and to some extent insulin alone, maintained normal and hyperplastic mouse mammary lobules in organ culture in a defined medium.

I am grateful to Drs. H. A. Bern and K. B. DeOme for suggestions, and to Victor Duran for photography.

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Prevention by Intravenous Injection of Antigen and Antibody of Passive Arthus Reaction to Unrelated Immune System. (24996)

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Previous work has shown that intravenous injection of antigen followed by its specific antibody resulted in marked decrease of passive cutaneous anaphylactic (PCA) response to an unrelated immune system in the rat(1). The possible dependence of this suppressive effect upon adequate levels of circulating complement (C′) has been suggested(2), since intensity of PCA paralleled *in vivo* fixation of C′ under a variety of experimental conditions. The present report concerns observations along similar lines in relation to direct Arthus reactions passively induced in guinea pigs.

Materials and methods. Antigens and antisera. The following antigen preparations were employed: 1. Bovine serum albumin (BSA), crystallized, obtained from Pentex, Kankakee, Ill., used without further purification. 2. *Chicken egg albumin* (Ea), recrystallized 5 times, prepared by method of Kekwick and Cannan(3). 3. *Rabbit anti-BS,* lots 23, 24, 27 and Pool I, '58, containing 0.479, 1.080, 0.263, and 0.362 AbN/per ml respectively. 4. *Rabbit anti-Ea,* lots 11, 15, and 17, containing 0.746, 1.412, and 1.197 mg AbN/per ml respectively. Antibody content of antisera was determined by quantitative precipitin method described in reference 4. *Complement titrations.* Serum samples for C′ titrations were obtained from blood (1-2 ml) drawn by cardiac puncture. Sera were clarified by centrifugation at 0°C and stored at −20°C until assayed. Estimations of hemolytic activity were carried out as described (5). *Hematologic determinations.*[*] 2 ml samples of blood were obtained by cardiac puncture with silicone-coated syringe and needle and collected into siliconed vial containing 0.025 ml of 10% solution of sodium versenate (EDTA). Leucocyte counts were performed as usual. For platelet counts the method of Feissly and Ludin, modified by Rosenfeld(6) was adopted. Blood was diluted 1:200 in siliconed red cell pipette using as diluting fluid a solution containing 0.05% sodium EDTA, 0.2% NaCl, and 3% cocaine hydrochloride. After careful mixing of contents, pipettes were left ½ hour at room temperature, then mechanically shaken for 3 minutes. A counting chamber of Levy-Neubauer type was filled with hemolysed content of pipette after discarding first drops and the homogeneously distributed, non-agglutinated platelets were counted under phase microscope over area of 2 mm^2. *Passive Arthus reactions* were produced by injecting 0.100 - 0.400 mg AbN (in 1 ml) by intravenous route followed immediately thereafter or 30 minutes later by 0.025-0.100 mg antigen N (in 0.1 ml) into skin of abdominal area of guinea pigs of 240-260 g. Animals were killed 2 hours after intradermal injection of antigen and reactions were read on undersurface after reflecting abdominal skin. Intensity of reaction was graded as recommended in(7), according to diameter of confluent or petechial hemorrhagic area: ++++ (more than 20 mm), +++ (15-20 mm), ++ (10-15 mm), + (5-10 mm), ± (less than 5 mm), and 0 (no reaction). The following experimental model was used in experiments such as in Table I:

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[*] The authors are indebted to Drs. M. Lindenberg and P. Azevedo for carrying out hematologic determinations. The help of Dr. G. Rosenfeld is also gratefully acknowledged.

0 hr	Intrav. inj. of "desensitizing" immune-system.
½	Bleeding for estimating C′ titer.
¾	Intrav. inj. of "sensitizing" (Arthus-producing) antiserum.
1¼	Intradermal inj. of Arthus-producing antigen.
3¼	Reading of results.

Intrav. inj. were performed with short-beveled gauge 26 needles into superficial veins of hind foot or of ear.

Results. A. *Influence on Arthus responsiveness of C′-depletion by previous intravenous injection of antigen and antibody.* Table I summarizes data on magnitude of passive Arthus reaction to intravenously injected anti-Ea (0.400 mg AbN) and intradermally injected Ea (0.025 and 0.050 mg EaN) in 3 groups of guinea pigs intravenously inoculated 45 minutes before with 0.200 mg BSA N plus 0.400 mg anti-BSA N or with either one of preceding reagents. A fourth group of non-injected controls was also included. Reactions of maximal (++++) or sub-maximal severity (+++) were observed in all animals, except those injected with BSA plus anti-BSA in which a clear cut suppressive effect was obtained. Complement titers in last group corresponded to approximately 10% of units present in other groups.

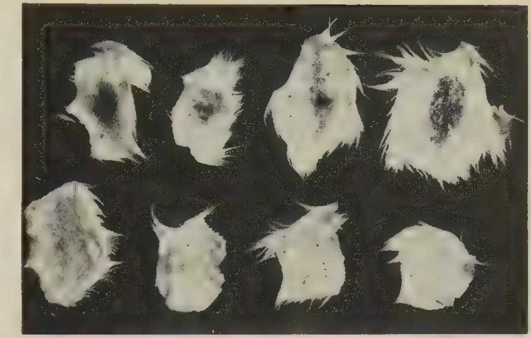

FIG. 1. Prevention by intrav. inj. BSA-anti BSA of Arthus reaction to intrav. inj. anti-Ea (.4 mg AbN) and intraderm. inj. Ea (.03 mg N). Skins in upper row are from non-desensitized guinea pig controls.

Identical results were obtained by using BSA-anti-BSA as C′-depleting system and Ea-anti-Ea as the Arthus producing system.

To investigate possible correlation between suppressive effect and complement drop effected by intravenous injection of antigen and antibody, an experiment was performed in which 3 different amounts of Ea/anti-Ea were intravenously injected one hour before sensitizing dose of anti-BSA. Pertinent data are

TABLE I. Prevention by Intravenously Injected BSA and Rabbit Anti-BSA of Direct Arthus Reactions in Guinea Pigs Sensitized with Rabbit Anti-Ea.

Amt of BSA and/or anti-BSA inj. intrav., mg N	Intensity of Arthus reaction 2 hr after intrad. inj. of Ea .050 mg N	.025 mg N	C′ titers
I. .20 BSA followed immediately by .40 anti-BSA	0	0	17
	±	±	26
	0	0	25
	±	±	25
II. .20 BSA	4+	4+	267
	4+	4+	310
III. .40 anti-BSA	4+	3+	214
	3+	3+	117
IV. None	4+	4+	247
	4+	4+	227

Animals sensitized with .40 mg anti-Ea N 45′ after intrav. inj. of BSA/anti-BSA. Bleedings performed 15′ before sensitization and intradermal inj. of Ea 30′ thereafter.

TABLE II. Amount of Intravenously Injected Ea and Anti-Ea Required to Prevent Passive Direct Arthus Reactions to BSA/Anti-BSA.

Amt of intrav. inj. Ea and anti-Ea, mg N	Intensity of Arthus reaction 2 hr after intrad. inj. of BSA		C′ titers
	.06 mg N	.03 mg N	
.20 Ea	0	0	20
.40 anti-Ea	0	0	16
	0	0	28
.10 Ea	0	0	48
.20 anti-Ea	±	0	40
	0	0	29
.05 Ea	±	±	118
.10 anti-Ea	+	+	85
	+	±	69
.40 anti-Ea	4+	3+	221
	2+	2+	207
	4+	3+	219

Animals sensitized with .40 mg anti-BSA N 1 hr after intrav. inj. of Ea/anti-Ea. Bleedings performed 20′ before sensitization and intrad. inj. of BSA immediately thereafter.

given in Table II. A very marked suppressive effect was obtained even in animals injected with 0.05 mg EaN plus 0.1 mg anti-EaN. C′ titers were normal in control group which received only 0.4 mg of anti-Ea N, whereas in other groups the reduction of C′ titer paralleled the amount of intravenously injected Ea/anti-Ea, *i.e.*, about 50% of normal titer in animals which received least amount of C′-depleting immune system.

Another way of investigating possible dependence of C′ levels upon suppressive effect described here, was to study the influence of previous Arthus reaction in animals sensitized by intravenous injections of 2 different antibodies. Table III reproduces results obtained in 2 groups of guinea pigs which received a mixture of 0.4 mg anti-Ea N + 0.4 mg anti-BSA N by intravenous route and tested intradermally 30 and 90 minutes later with each of corresponding antigens (0.100 mg N) in alternate order.

Table III shows that the first reaction was always of greater severity than the second. However, although a complement reduction to approximately 20-40% of normal titer was observed as a consequence of first antigen-antibody reaction, only a slight reduction in severity of second reaction was noted, particularly in the group in which Ea was injected before BSA. These results are contrasted with those given in Table II in which a very marked suppressive effect was detected even in presence of 69 to 118 units of C′.

The possibility of restoring Arthus responsiveness in C′ depleted animals by injecting fresh guinea pig serum was also investigated. Two groups of guinea pigs were decomplemented by intravenous injection of Ea/anti-Ea. Animals of one of the groups were "recomplemented" with 5 ml of fresh guinea pig serum and passive Arthus reactions were provoked in both groups by intravenous injection of 0.4 mg anti-BSA N followed by intradermal injection of 0.06 mg BSA N, 90 minutes after decomplementation.

Results showed that the suppressive effect could not be overcome by recomplementation, although C′ titers rose from 49-61 $C'H_{50}$ in the decomplemented group to 124-142 units in recomplemented animals.

B. *Duration of suppressive effect and its relationship to number of circulating leucocytes and platelets.* Since *in vivo* C′ fixation is accompanied by marked leucopenia(8), it was of interest to study variation in number of circulating leucocytes and platelets in relation to time of duration of the suppressive effect. Repeated experiments showed that the suppressive effect produced by intravenous injection of antigen and antibody is still very marked after 3 hours. After 6 hours, reactions were usually of same severity in decomplemented and control groups.

Experiments were performed to follow the

TABLE III. Severity of Arthus Reactions in Guinea Pigs Injected Simultaneously with Anti-Ea and Anti-BSA by Intravenous Route and Tested Intradermally 30 and 90 Minutes Later with Each of Corresponding Antigens in Alternate Order.

Order of intrad. inj.	Reactions 3 hr after 1st intrad. inj.		C′ titers
	.10 mg Ea N	.10 mg BSA N	
Ea—BSA	2+	±	42
	4+	2+	77
	4+	3+	
	4+	3+	
BSA—Ea	±	4+	53
	2+	4+	113
	2+	4+	82
	2+	4+	

Intrav. inj. of mixture of .40 mg anti-Ea N plus .40 mg anti-BSA N. Bleedings 10′ before second intrad. inj.

TABLE IV. Effect of Intravenously Injected Antigen and Antibody on Circulating Leucocytes and Platelets.*

Group	Time between inj. of BSA/anti-BSA and bleeding for leucocyte and platelet counts (hr)	Total leucocytes/mm^3	Platelets/mm^3 × 1000
A. Inj. intrav. with .2 mg anti-BSA N followed by .4 mg BSA N	None	980	0
		680	0
	2	9720	148
		6300	94
	4	6500	164
		8580	146
	6	6400	142
		8740	301
B. Non-injected controls	—	9720	346
		5510	324

* Results above refer to individual animals.

number of circulating leucocytes and platelets at 0, 2, 4, and 6 hours after decomplementation. The number of circulating leucocytes falls to approximately 10% immediately after intravenous injection of antigen and antibody but becomes normal again after 2 hours (Table IV). The effect on platelets is more striking and recovery slower, approaching normal values after 6 hours.

Discussion. The fact that no correlation was found between lack of Arthus response and number of C′ units in circulating blood when the Arthus-producing system was injected (Cf Tables II and III), seems to indicate that C′ depletion is not a causal factor in abolishment of the reaction.

Stone(9), pointing out that guinea pigs are difficult to decomplement by injection of antigen-antibody combinations, also reached the conclusion that C′ was not the critical factor in his experiments on inhibition of Arthus reaction during protracted anaphylactic shock.

Since "desensitizing" effect of C′-depleting immune system may depend on a critical concentration of certain C′-component(s) not expressed by whole hemolytic titers as in present experiments. Corroborating evidence against the rôle of C′ is given by failure of recomplementation in restoring Arthus reactivity in C′-depleted animals.

The possible rôle of platelets is more strongly suggested. Immune adherence of platelets on specific precipitate used for "desensitization" would result in a transitory deficiency of these elements at site of intradermal injection of antigen and thus prevent local vascular effects essential for development of the hemorrhagic reaction. This would be in logical agreement with Stetson's interpretation(10) of the mechanism of Arthus and Shwartzman reactions.

It is apparent, however, that a final explanation of our findings awaits further investigations.

Summary. 1. Previous intravenous injection of antigen and antibody prevents development of passive Arthus reaction to an unrelated immune system in guinea pigs. 2. This suppressive effect does not seem to depend on C′ depletion but rather on deficiency of platelets in circulating blood.

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Effects of Air Ions on Trachea of Primates.* (24997)

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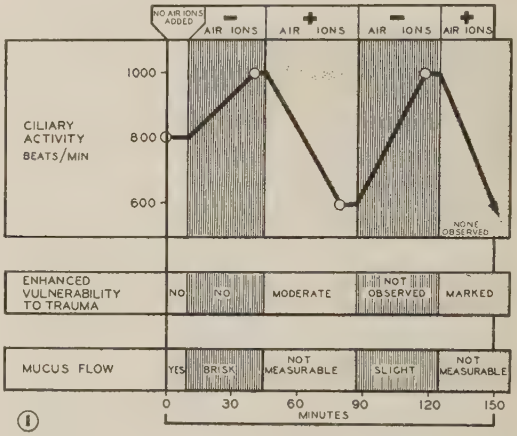
It has been shown that positive air ions reduce the rates of ciliary beat and mucus flow in the extirpated tracheal strip, while negative ions accelerate both processes(1). Direct administration of positive air ions to tracheal mucosa *via* trachectomy aperture produces identical inhibition of ciliary activity and in addition increases vulnerability to trauma of mucosal blood vessels. Exposure to negative air ions has the opposite effect(2). Comparable results follow inspiration of air containing relatively large numbers of unipolar air ions by unoperated, ambulatory mice. These effects were observed in rats, mice, guinea pigs and rabbits. In this paper we describe the action of air ions on tracheas of monkeys and man.

Methods. Monkey trachea. Three healthy cynomolgus monkeys were anaesthetized with sodium pentothal. In each case the trachea was carefully exposed, an oblong portion of ventral surface measuring 10 mm by 5 mm was outlined with high frequency desiccating current applicator and then removed with scissors. In 2 instances a copper terminal wound with gauze saturated with Ringer's solution was used as ground of ion generator's rectifying circuit and placed in contact with the trachea just caudal to tracheal window; in one case no ground was used. The monkey's head and thorax were enclosed in glass and plastic chamber to permit microscopic observation of tracheal surface during exposure to air ions in an atmosphere maintained at approximately 25°C with relative humidity Ca. 90%(2). *Tracheal sections* were obtained at autopsy from 2 patients free from respiratory disease and kept in a moist chamber until used. In one case the time from death to beginning of experiment was 5 hours, and in the other 6½ hours. Strips were cut from the trachea and placed in humid air chamber following technic previously described(1), except that tissue strips were ungrounded. *Small air ions* were generated by beta radiation emitted by tritium contained in sealed foils. A reversible rectifying circuit permitted selection of positive or negative ions (3). Measurements made with Beckett probe and Beckman micromicroammeter established that Ca. 1×10^9 air ions of either charge im-



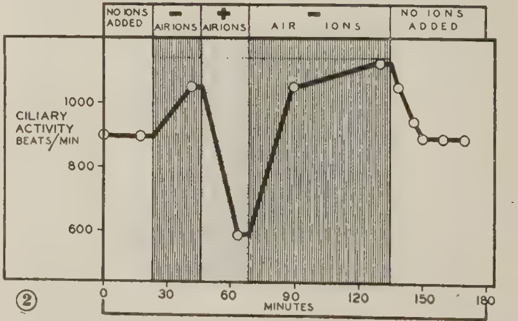


FIG. 1. Effects of unipolar small air ions on trachea of living cynomolgus monkey. Ions administered via tracheotomy aperture.

FIG. 2. Effects of unipolar small air ions on extirpated human trachea. (Strips removed post-mortem.)

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† NAMRU #1-NBL signifies Naval Mediral Research Unit No. 1 and Naval Biological Laboratory. The opinions expressed are not necessarily those of Navy Department.

pinged on each cm^2 of exposed tissue/second with grounds in place. Ion densities were only slightly less when tissues were ungrounded. Ciliary rates were determined with a strobotachometer(1).

Results. Fig. 1 typifies results obtained with the 3 monkeys. As is true with mice, rats, guinea pigs and rabbits, negative air ions enhanced the efficiency of the tracheal surface clearing mechanism while positive ions depressed it. In tissues untreated with air ions the minor trauma produced by touching the surface with a probe induced no more than a brief vascular reaction, but after exposure to positive ions this procedure resulted in prompt production of a well-defined ecchymotic area. Grounding the rectifying circuit did not measurably influence the tissue response.

In both human specimens the layer of mucus was minimal and it was not possible to measure rates of mucus flow. However, the characteristic effects of air ions on ciliary activity were well defined in each instance. As indicated in Fig. 2, treatment with negative ions raised rate of beat, and it quickly returned to normal when ion source was removed. Positive ions depressed the rate and this depression was reversible.

Conclusions. Positive air ions depress ciliary activity of trachea of monkeys and man, while negative ions increase it.

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Carcass Weight Changes in Rats Bearing Walker Carcinosarcoma 256.
(24998)

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Liver hypertrophy has been frequently observed in tumor-bearing rodents(1) and enlargement was an actual increase in liver substance by nitrogen(2), moisture contents(3) and increased mitotic activity(4). Yeakel(5) suggested that this hypertrophy may be due to increased protein anabolism necessary for neoplastic tissue synthesis. Since liver enlargement occurred when dietary intake was inadequate to furnish the needs of the neoplasm, various tissues relinquished nitrogen to the metabolic pool(2). If these suggestions are correct, partial removal of liver during this period might be expected to inhibit tumor growth and affect changes in carcass weight. In the present experiments, animals were partially hepatectomized during period of rapid tumor growth and changes in body weight as well as tumor growth were followed.

Methods. Female Holtzman rats received intramuscular injections(6) of a suspension of Walker carcinosarcoma 256. Initial weight of animals was 170-190 g. They were fed Purina laboratory chow and water *ad lib.* Partial hepatectomies (70% of liver removed) were performed by the method of Higgins and Anderson(7) on 7th day of tumor growth when tumors weighed 0.5-2. g. Our preliminary experiments had shown that splenectomy, as well as partial hepatectomy, resulted in larger final liver mass in rats bearing Walker tumor. A splenectomized group was therefore included in the experiments. This operation was performed 2 days before tumor implantation.

Results. Prior to studies on partial hepatectomy, liver weights and carcass weights (total body weight minus tumor weight) were determined in 62 tumor bearers. Animals were sacrificed after 19 days of tumor growth when tumor weights ranged 40-60 g. Fig. 1 shows relation of liver weight to carcass weight changes. Rats with large livers gained carcass weight while those with small livers lost weight and there was a close correlation between those two factors ($r = 0.92$).

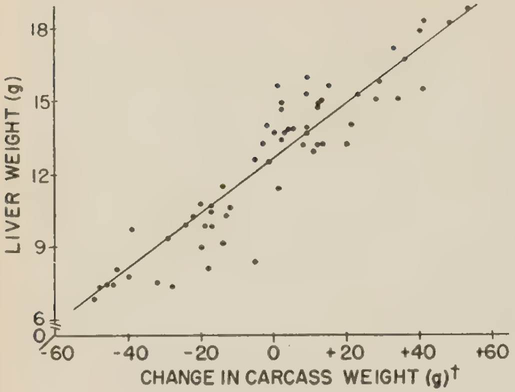

FIG. 1. Relationship of liver wt and carcass wt gains in tumor-bearing rats.*

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* Walker carcinosarcoma 256 weighing 40-60 g.
† Carcass wt is total body wt minus wt of tumors.

Generally liver moisture contents of tumor bearers are higher than those of normal animals, but there was no appreciable difference in moisture content of tumor bearers with large and small livers. For example, moisture content of livers of tumor bearers was 76.2 ± .1% as compared to 71.7 ± .2% for normal animals of similar size, but percent liver moisture of tumor bearers with large and small livers was 76.2 ± .2 and 76.1 ± .2 respectively.

Table I shows relationship of splenectomy and partial hepatectomy to tumor and carcass weight changes. All animals were sacrificed after 14 days of tumor growth. Apparently splenectomy and partial hepatectomy had no effect on final tumor weights. Final liver weights of both splenectomized and hepatectomized groups were significantly higher than control groups. Nevertheless, the only treatment resulting in carcass weight gain was partial hepatectomy which was associated with an average weight gain 22 g more than any other group. Our unpublished experiments on 30 normal animals showed no difference in 16-day weight gains between partially hepatectomized (48 ± 5 g) and sham operated animals (45 ± 6 g).

Discussion. Results of initial experiments indicated at least 2 possible relations between liver and carcass weight changes in tumor bearers, *i.e.*, (A) A larger final liver mass enhanced carcass weight gains, or (B) A greater growth rate of liver during period of rapid tumor growth enhanced carcass weight gains. The first possibility must be eliminated since the splenectomized group had final liver weights equivalent to partially hepatectomized animals but exhibited a loss in carcass weight. The second possibility was reasonable assuming splenectomy did not inhibit carcass weight gains. Unpublished experiments on 14 normal animals indicated that splenectomy had no effect on changes in carcass weight over a 16-day period. Therefore, increased carcass weight appeared to be related to growth rate of liver in partially hepatectomized group rather than final liver mass.

Splenectomy and partial hepatectomy had no effect on final tumor weights. These latter results may appear to be in contrast to those of Paschkis, *et al.*(8) who reported that partial hepatectomy accelerated tumor growth. However, partial liver removal was performed at time of tumor transplantation and not at time of rapid tumor growth. Partial hepatectomy therefore probably decreased the period

TABLE I. Effect of Partial Hepatectomy and Splenectomy upon Carcass Weight Gains in Tumor-Bearing Rats.*

Treatment	No. rats	Tumor wt (g)	Liver wt (g)	Change in carcass wt (g)
Control	13	34 ± 2§	9.0 ± .4	−16 ± 6
Sham splenectomy†	12	36 ± 2	9.1 ± .3	−19 ± 6
Splenectomy†	12	40 ± 2	11.5 ± .4‖	−15 ± 7
Sham hepatectomy‡	13	35 ± 2	9.8 ± .3	−12 ± 8
Partial " ‡	13	36 ± 2	11.8 ± .4‖	+10 ± 1‖

* 14-day-old Walker carcinosarcoma 256.
† 2 days before tumor implantation.
‡ On 7th day of tumor growth.
§ Mean ± stand. error.
‖ Significantly different from sham (P <.01).

of "tumor take," which would result in larger final tumor weight.

Since Miller, Fancher and Bale(9) reported that complete removal of liver would inhibit incorporation of lysine-C^{14} into Walker tumor, and in view of our studies it seemed a small amount of liver tissue could provide the necessary nutrients for neoplasm even during period of rapid tumor growth. Under these conditions, partial hepatectomy did not affect final tumor weight but did enhance carcass weight gains.

Summary. Liver weights of rats bearing 40-60 g Walker carcinosarcoma 256 tumors were directly correlated with changes in carcass weight of host. Removal of 70% of liver during rapid growth of tumor resulted in a gain in carcass weight without affecting growth of the tumor.

Authors are indebted to Mike Brown and Mabelle Adams for performing hepatectomies and to Wilma Goodwin and Faye Jones for technical assistance.

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Antigenicity of Connective Tissue Extracts. (24999)

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The immunologic concept of pathogenesis of "collagen diseases" has stimulated interest in studies of antigenicity of connective tissue and its components. Such investigations resulted only rarely in successful production of specific antibodies. Watson, Rothbard and Vanamee(1) induced complement fixing antibodies to acetic acid extracts of rat collagen and concluded that these antibodies were specific to collagen. Attempts to demonstrate antigenicity of hyaluronic acid(2) and chondroitin sulphate(3) have not been successful. In previous experiments, reported in abstract only(4), injection of saline extracts from rabbit tendons into guinea pigs did not elicit anticonnective tissue antibodies, but in serum of injected guinea pigs high levels of complement fixing antibodies to saline extracts of rabbit and human leukocytes were demonstrated. This apparent experimental immunologic relationship of connective tissue and leukocyte extracts was of great interest in view of immunologic abnormalities in acute disseminated lupus erythematosus. When other batches of tendon extracts failed to stimulate generation of such antileukocytic antibodies a search was made for the cause of this variability in antigenic behavior of saline extracts from rabbit tendons. Results of the present study indicate that neutral salt extracts from leg tendons of aged rabbits, combined with Freund's adjuvant, elicit a greater antibody response in guinea pigs than such extracts from tendons of young animals. In contrast to previous experiments anticonnective tissue antibodies were demonstrable. These anticonnective tissue sera also crossreacted with the cytoplasmic fraction of rabbit leukocytes.

Methods. Extracts of connective tissue were prepared as follows: Tendons from small muscles of lower extremities of 7-day-old and aged rabbits (older than 1 year) were carefully separated from muscle tissue, washed in running tap water, soaked in cold tap water overnight and transferred into physiologic saline solution* (10 ml/g wet tissue). They were then ground 10 minutes in cooled Waring blendor. To achieve a final protein concentration of 150 μg/ml (determined according to method of Lowry(5)) the filtrate was either diluted with saline or concentrated by dialysis against a 30% solution of Polyvinylpyrrolidone. This material was then either used fresh or stored at –20°C for later use. The filtrate was mixed with equal amount of Freund's adjuvant. Aliquots of 1 ml of this mixture were injected subcutaneously or intramuscularly into multiple sites of male guinea pigs of Hartley Strain, weighing approximately 250 g. These injections were given at weekly intervals for total of 4 ml (300 μg of protein). Blood was obtained by cardiac puncture 10 days after final injection. Thirty-one guinea pigs were injected with connective tissue extracts obtained from old animals (subsequently called "O.C.T.") and 32 animals were injected with such extracts obtained from young animals (subsequently called "Y.C.T."). Three animals were injected with Freund's adjuvant alone and 5 with rabbit serum. Leukocytic extracts were prepared according to method of Miescher(6) which permits separation into cytoplasmic and nuclear fraction. The cytoplasmic fraction was then dialyzed against distilled water to remove sucrose and citric acid from original extract and adjusted to isotonicity by addition of sodium chloride and further diluted, if necessary, with physiologic saline to result in protein concentration of 150 μg/ml. The nuclear fraction, soluble in 1 M saline, was adjusted to concentration of 150 μg/ml by addition of distilled water until isotonicity was achieved. If further dilution was necessary for desired concentration, saline was used. In the process of dilution the nuclear material precipitated, but could be maintained in suspension. Two groups of 5 animals each were injected with these fractions prepared from rabbit leukocytes. All substances with exception of adjuvant were also used as test antigens in the complement fixation test, performed according to standard method of Kolmer. For demonstration of precipitins the agar double diffusion technic of Ouchterlony as modified in this laboratory(7) was used. Statistical analysis was performed by chi-square method following grouping of results into 4-fold contingency tables.

TABLE I. Complement Fixing Antibodies to "Y.C.T." and "O.C.T."

Titer*	No. of animals sensitized with "Y.C.T."	"O.C.T."
<8	21	9
>8	11	22

* "O.C.T." used as test antigen; "Y.C.T." ineffective as test antigen (Fig. 1).

$X^2 = 7.3$; $P < .01$.

Results. The majority of guinea pigs injected with "Y.C.T." developed complement fixing antibodies of low titer (Fig. 1, Table I) with the use of "O.C.T." as test antigen. With "Y.C.T." as test antigen the titers were even smaller, only 3 out of 32 guinea pigs had antibody levels at 8 and 16 fold dilution. Thus, "Y.C.T." was not only a poor sensitizing but also a poor test antigen.

In contrast, 22 out of 31 guinea pigs injected with "O.C.T." had antibody levels demonstrable at higher than 16 fold dilution (Fig. 1). The difference in antigenicity between "O.C.T." and "Y.C.T." was statistically significant at the 1% level (Table I).

No complement fixing antibodies to connective tissue extracts could be found in animals injected with Freund's adjuvant alone or with

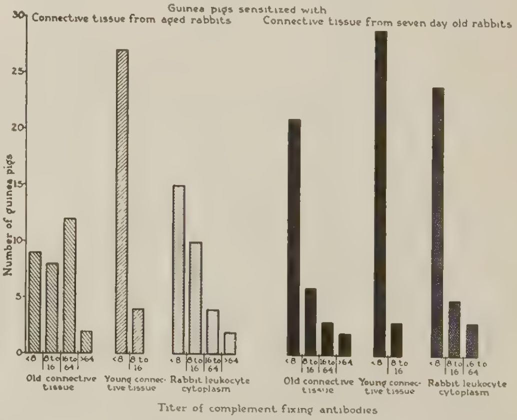

FIG. 1. Levels of antibodies to "O.C.T." and "Y.C.T." (see text for explanation).

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* Saline solution used in immunology laboratory contains 0.85% sodium chloride, 0.01% magnesium sulphate, and 0.004% calcium chloride.

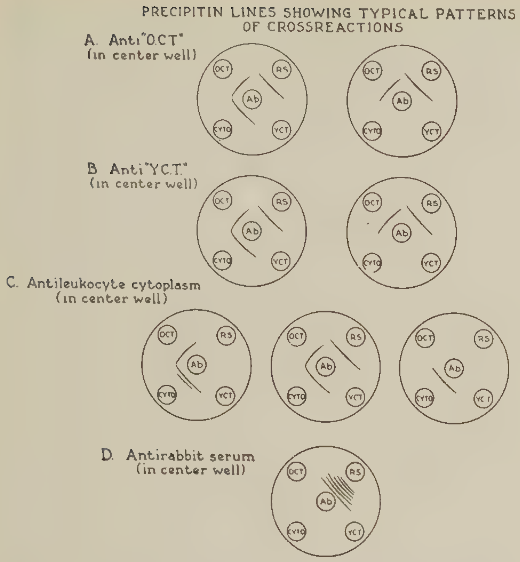
PRECIPITIN LINES SHOWING TYPICAL PATTERNS OF CROSSREACTIONS
A. Anti "O.C.T." (in center well)
B. Anti "Y.C.T." (in center well)
C. Antileukocyte cytoplasm (in center well)
D. Antirabbit serum (in center well)
OCT
RS
Ab
CYTO
YCT

FIG. 2. Precipitin lines in double diffusion agar. Ab—Antirabbit serum, OCT—''Old'' connective tissue, YCT—''Young'' connective tissue, CYTO—Leukocyte cytoplasmic extract, RS—Native rabbit serum.

serum. No precipitins were demonstrable in the test tube on overnight incubation, but with agar double diffusion technic precipitin lines were present between "O.C.T." as test antigen and antisera to "O.C.T." (Fig. 2A). Such precipitins were also seen between "O.C.T." as test antigen and antisera to "Y.C.T.", but no precipitins occurred when "Y.C.T." was used as test antigen. As with the complement fixing test "Y.C.T." was also a poor test antigen for the demonstration of precipitating antibodies.

A precipitin reaction was also noted between antisera to "O.C.T." and native rabbit serum (Fig. 2A). On the other hand no such reaction was seen between guinea pig serum containing anti-rabbit serum antibodies and "O.C.T." or "Y.C.T." (Fig. 2D).

Animals injected with "O.C.T." and "Y.C.T." developed complement fixing and precipitating antibodies to leukocyte cytoplasm (Figs. 1, 2). In the complement fixation test higher antibody levels were observed in guinea pigs sensitized with "O.C.T.", but the difference between these animals and those injected with "Y.C.T." was not statistically significant ($0.1 > P > 0.05$, Table II). No complement fixing antibodies to nuclear antigen could be demonstrated in these guinea pigs.

TABLE II. Production of C.F. Antibodies to Leukocytic Cytoplasm with ''Y.C.T.'' and ''O.C.T.''

Titer	No. of animals sensitized with ''Y.C.T.''	 ''O.C.T.''
<8	24	15
>8	8	16

$X^2 = 3.8$; $P > .05$ (for $P = .05$, X^2 of 3.84 would be required).

In sera of animals injected with leukocyte fractions, complement fixing antibodies to the respective fractions were demonstrated, with slight crossreaction between cytoplasmic and nuclear material. In the Ouchterlony preparation, however, crossreaction of anticytoplasmic antibodies with "O.C.T." was apparent. They also reacted with rabbit serum (Fig. 2C).

Guinea pigs injected with connective tissue extracts failed to gain weight and their fur became sparse (Fig. 3). Pathological findings in these animals are being investigated. There was apparently no difference in this reaction whether animals were injected with "O.C.T." or "Y.C.T.". No animals injected with the other substances exhibited these alterations.

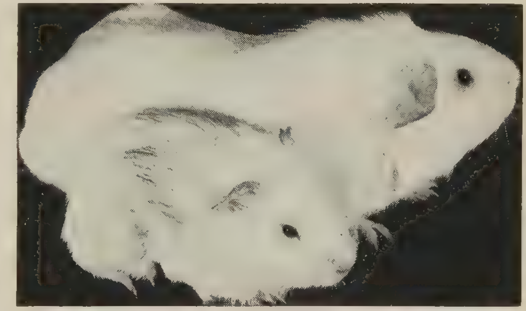

FIG. 3. Appearance of guinea pigs 7 wk after start of experiment. Rear—Control animal. Front—''O.C.T.'' inj. guinea pig.

Studies on chemical characterization of the antigenic component of connective tissue extracts are in progress. Preliminary observations indicate presence of hydroxyproline in hydrolysates of these connective tissue extracts.

Discussion. Tendons consist predominantly of 25-30% collagen and 0.75-1.5% of mucoproteins(8). The bulk of the remainder is water. According to Meyer(9) mucopolysaccharides of the ground substance of tendons consist predominantly of chondroitin sulphate B and C which are firmly bound to protein. Chondroitin sulphate B cannot be extracted with water or neutral salt solutions which were used in our experiments. Chondroitin sulphate C is slightly more soluble in such neutral salt solutions. Collagen is not only the protein of fibrils, but a fraction of it appears dispersed in the ground substance (10) and can be extracted with neutral salt solutions of low molarity. This neutral salt soluble fraction of collagen is small, except in actively growing tissue(11) and in connective tissue stimulated into active growth by carrageenin(12). All connective tissue extracts used contained the same amount of protein. Nevertheless, there was a marked difference in antigenicity between connective tissue extracts from old and those from young rabbits. The cause of this is still obscure. It is possible that because of lower solubility of "adult" collagen in neutral salt solutions a larger amount of it had to be extracted to yield 150 μg of protein/ml than from "young" connective tissue. This could have resulted in a higher concentration of substances which might be responsible for the greater antigenicity. It is not likely that these substances are mucopolysaccharides as it has been shown (13) that concentration of sulphated polysaccharides in neutral salt solutions of connective tissue is directly proportional to concentration of neutral salt soluble fraction of collagen.

The difference in antigenicity between "O.C.T." and "Y.C.T." was demonstrated mainly in the complement fixation test. On the other hand "young" connective tissue stimulated development of precipitins in sera of injected guinea pigs as well as "O.C.T.", but these precipitins could only be demonstrated with "O.C.T." as test antigen.

In the precipitin test antisera to connective tissue preparations also reacted with serum. Could this crossreaction with serum be interpreted as serum contamination of connective tissue extracts? This is not likely. Native tendons contain only a small amount of plasmaproteins(8), and moreover it is likely that this amount was further reduced by preparation of the extracts. The fact that anti-rabbit-serum antibodies did not react with connective tissue extracts, is not consistent with the possibility of presence of rabbit serum in these extracts. It is more likely that the rabbit serum contained a small amount of antigenic substance of connective tissue extracts.

The crossreaction of anticonnective tissue antibodies with rabbit leukocyte cytoplasm is of great interest. In previous experiments antileukocytic antibodies were demonstrated in guinea pigs sensitized with connective tissue extracts(4). As test antigen saline extracts of rabbit and human leukocytes were used. No attempt was made to separate leukocytes into cytoplasmic and nuclear fractions. In our experiments it could be shown that only cytoplasmic fraction of leukocytes reacted with anticonnective tissue sera. The failure to demonstrate antigenicity of nuclear extracts in the precipitin test seems relatable to their insolubility in physiologic saline and to resulting incapacity to diffuse in agar gel. In the complement fixation test, however, this technical problem does not exist, as evidenced by the demonstration of homologous antinuclear complement fixing antibodies. The fact, therefore, that in this test anticonnective tissue sera did not react with nuclear extracts, speaks in favor of a lack of an immunologic relationship between extracts of leukocyte nuclei and connective tissue.

Preliminary observations indicate presence of hydroxyproline in hydrolysates of cytoplasmic extracts of rabbit leukocytes. This could permit the conclusion that these leukocytes contain collagen. It remains to be proved whether this explains the apparent immunologic relationship between connective tissue and leukocyte cytoplasm.

Summary. 1. Antibodies to rabbit connective tissue have been induced in the guinea pig. 2. Connective tissue extracts from aged rabbits had greater antigenicity than from young animals. 3. Anticonnective tissue serum crossreacted with the cytoplasmic fraction of rabbit leukocytes. 4. Chemical characteristics of the antigenic component of connective tissue remain incompletely explored.

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Disease in Macacus Monkeys Inoculated with ECHO Viruses.* (25000)

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Several ECHO viruses have been implicated as causative agents of neurologic disease in man(1). Paralysis has occurred in individual patients infected with some of these viruses(2,3,4,5). The recent reports of Steigman(6), Dalldorf(7) and of Habel and Loomis(8) leave no doubt that paralysis and neuronal damage can be induced in monkeys experimentally infected with Coxsackie viruses representative of both A and B groups. These investigators observed microscopic changes in the central nervous system of inoculated animals which were indistinguishable from those caused by poliovirus. Wenner and Chin(9) in monkeys used for preparation of antiserum against ECHO prototype strains described degenerative changes in neurons after the animals had received several injections of virus and mineral oil adjuvant. Our experiments were undertaken to determine whether 2 types of ECHO virus could produce disease of the central nervous system in monkeys.

Materials and methods. Tissue cultures. Cultures were prepared according to previously described technics(10,11). Cultures of human amnion cells were used in attempts to demonstrate virus in materials derived from inoculated monkeys and in neutralization tests with their serum. The *viruses* employed were ECHO Type 6, strain SHO(2) and ECHO Type 16, strain HAR(2). Clinical manifestations in patients infected with these 2 strains have been described by Kibrick *et al.*(2). The strains were recovered in tissue culture from fecal specimens. The type 6 strain was isolated in human kidney cells and subsequently passed once in this medium and twice in human amnion cells. The fluid from the last of these passages had an infectivity titer of $10^{-6.5}$/0.1 ml in amnion cells and was used as inoculum in the experiments to be described. The type 16 strain was recovered in human kidney cells, passed twice in human embryonic skin and muscle tissue, and twice in human amnion cells. The fluid from the last of these passages had an infectivity titer of $10^{-3.5}$/0.1 ml in cultures of human amnion cells, and was used as inoculum. *Monkeys.* Seven rhesus and 3 cynomolgus monkeys were studied. Before each was injected, specimens of blood for antibody determination, heparinized blood for viral culture, and peripheral blood for white blood cell counts were obtained. Then a #22 gauge hypodermic needle was introduced into the basal cistern, and about 1-2 ml of cerebrospinal fluid was withdrawn for white cell count and for viral culture. Eight of the monkeys were inoculated with 0.5 ml of undiluted tissue culture fluid containing the virus which was introduced into the basal cistern. In half of the animals

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† Supported by fellowship from Commonwealth Fund. Present address: Univ. of North Carolina School of Med., Chapel Hill.

0.1 ml of the fluid was also injected intraspinously into the lumbar enlargement. Two animals serving as controls were inoculated with infected tissue culture fluid that had been heated at 65°C for ½ hour. No active virus was demonstrated in these fluids upon subsequent addition to cultures of human amnion cells. To one control animal 0.5 ml of heated ECHO 16 virus was administered intrathecally and 0.1 ml intraspinously; to the other 0.5 ml of heated ECHO 6 virus was given intrathecally. Specimens of blood and spinal fluid for leucocyte counts and for detection of virus, and samples of feces for detection of virus were obtained every other day during the first week and every third day thereafter until termination of experiment. The animals were examined daily for muscle weakness or paralysis. At intervals varying from 6-21 days after injection, the animals were sacrificed. The brains and spinal cords were immediately removed; portions were preserved in the CO_2 cabinet to test later for presence of virus. The remainder of each tissue was fixed in 10% formalin. *Neutralization tests.* Following centrifugation serum was removed from the clotted blood and stored at −20°C until tested. Undiluted serum was inactivated at 56°C for one half hour, then a series of dilutions, increasing by a factor of 2, was prepared. To each serum dilution was added an equal volume of suspension which contained virus in approximately 100 $TCID_{50}$/0.1 ml. The mixture was incubated at 4°C for one hour, at which time 0.2 ml of the virus-serum mixture was added to each of 3 tubes containing cultures of human amnion cells. Control tubes containing cultures inoculated with a diluted suspension of virus only and uninoculated cultures were included in each test. The neutralization titer of a serum was determined as the reciprocal of that dilution which prevented the appearance of cytopathic effect for 14 days.

TABLE I. Results of Tests for Presence of ECHO Virus.

Day after inj.	Source of specimen	No. of animals* Type 6 virus	Type 16 virus	Control
2	Blood	4/4	0/4	0/2
	CSF	"	3/4	"
4	Blood	0/4	0/4	"
	CSF	4/4	1/4	"

* Numerator = No. positive for virus. Denominator = No. tested.

TABLE II. Neutralizing Antibody Response to Injected Virus.

Virus	Monkey	Before inj. Day	Titer*	After inj. Day	Titer*
ECHO 6	control 1	0	<2	8	<2
	2	"	"	21	64
	3	"	"	"	"
	4	"	"	8	32
	5	"	"	"	16
ECHO 16	control 6	"	"	21	2
	7	"	"	"	32
	8	"	"	"	16
	9	"	"	6	4
	10	"	"	"	"

* Reciprocal of final serum dilution.

Results. The results of tests for presence of virus in blood and spinal fluid are shown in Table I. From blood of the 4 animals injected with unheated type 6 virus the agent was recovered on second day but not thereafter. The presence of virus was also detected in the cerebrospinal fluid in specimens obtained on the second and fourth days after inoculation. The agent was not demonstrated in blood of any of the 4 animals injected with active ECHO 16 virus although in all but one instance virus was recovered from the cerebrospinal fluid. Virus was not detectable in *22* blood specimens and *22* cerebrospinal fluid specimens collected between sixth and twenty-first days. No virus was detected in stools of any of the monkeys, nor in specimens of tissue taken at autopsy.

That infection occurred in these animals is also indicated by development of neutralizing antibodies to the injected type of virus. The undiluted serum from all animals obtained at

TABLE III. Muscular Weakness in Relation to Infection with ECHO Virus.

Extremities involved Leg	Arm	No. animals	ECHO virus Type 6	Type 16
—None—		2	1	1
1		1		1
2		1		1
1 +	1	1	1	
2 +	1	3	2	1
Total		8	4	4

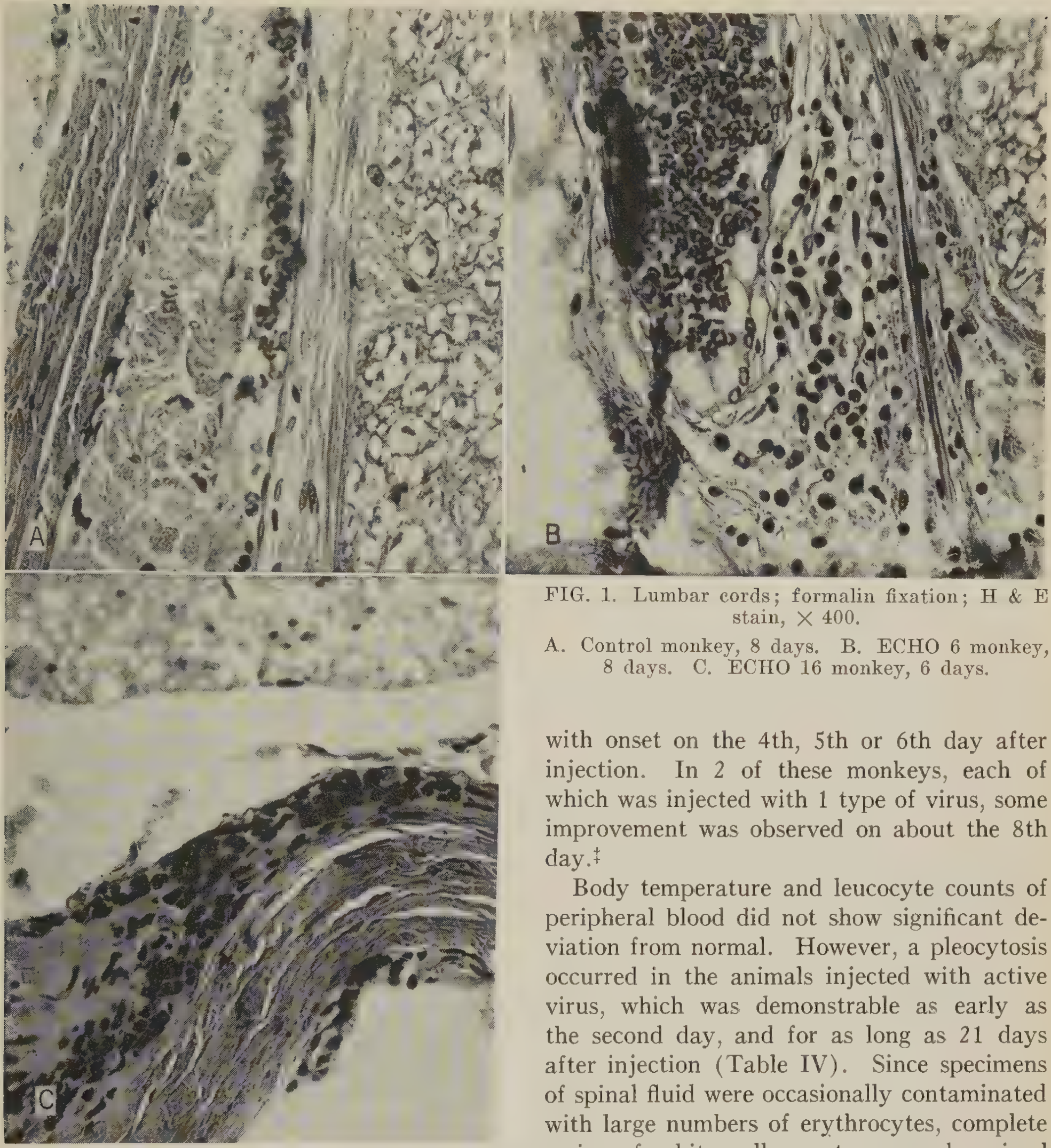

FIG. 1. Lumbar cords; formalin fixation; H & E stain, × 400.

A. Control monkey, 8 days. B. ECHO 6 monkey, 8 days. C. ECHO 16 monkey, 6 days.

time of injection failed to neutralize approximately 100 ID_{50} of the homologous virus. A significant increase in homologous antibody occurred in all the monkeys injected with active type 6 virus (Table II). A significant antibody response also occurred in animals injected with active type 16 virus (Table II).

The incidence and location of muscular weakness are recorded in Table III. Three of the animals injected with active ECHO 6 and 3 of those injected with active ECHO 16 developed definite signs of muscular weakness, with onset on the 4th, 5th or 6th day after injection. In 2 of these monkeys, each of which was injected with 1 type of virus, some improvement was observed on about the 8th day.‡

Body temperature and leucocyte counts of peripheral blood did not show significant deviation from normal. However, a pleocytosis occurred in the animals injected with active virus, which was demonstrable as early as the second day, and for as long as 21 days after injection (Table IV). Since specimens of spinal fluid were occasionally contaminated with large numbers of erythrocytes, complete series of white cell counts on each animal cannot be presented. The cell count of the spinal fluid at its peak varied from 64 to 598 cells/mm^3. Variations of this order of magnitude were recorded in both groups of monkeys inoculated respectively with the 2 types of ECHO virus. In the cerebrospinal fluid of the 2 animals that received heated tissue

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‡ We are grateful to Dr. David Grice, formerly of Orthopedic Service, Children's Medical Center, for indispensable aid in evaluating muscle weakness in these animals.

TABLE IV. Leukocytes in CSF of Monkeys Inoculated with Active ECHO Viruses Types 6 and 16.

Cells/mm^3 CSF	No. of monkeys								
500-599			1		1				
400-499		2							
300-399		2							
200-299		1		1	2				
100-199		2	2						
21- 99		1	1	1	1	1	1	2	2
0- 20	8					1	3	2	2
	0	2	4	6	7	8	10	15	21
	Day after inoculation								

Maximal WBC count in 2 control monkeys receiving heat-inactivated viruses was 20/mm^3.

culture fluid, the highest count observed was 20 cells/mm^3.

Microscopic examination of the spinal cords revealed no convincing evidence of neuronal damage. In sections from certain monkeys that received active virus, however, slight round cell infiltration of the meninges was observed in a few areas (Fig. 1).§

Discussion. Mild or moderate muscle weakness has been observed in human beings infected with ECHO types 2(6), 4(4), 6(2), 9(5), and 16(4) virus. It is therefore of interest that in monkeys experimentally inoculated with ECHO 6 and 16 certain muscles were affected. The findings here reported also indicate that, just as in man, these 2 representative ECHO viruses are capable of producing in rhesus and cynomolgus monkeys a mild meningitis characterized by the presence of virus in the spinal fluid and a predominantly mononuclear pleocytosis.

Summary. In monkeys injected intrathecally with ECHO virus 6 and 16, clinical signs of muscle weakness, pleocytosis of cerebrospinal fluid and development of homologous neutralizing antibodies were observed. These reactions were not detected in 2 animals inoculated in comparable manner with suspensions of these viruses which had been heated at 65°C for one half hour.

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§ We are indebted to Doctors D. E. Denny-Brown, Joseph M. Foley and John M. Craig for examining sections of CNS tissues.

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Lipemia-Induced Acceleration of Intravascular Clotting.* (25001)

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Reports of changes in coagulability of blood associated with post-prandial lipemia have thus far been based on test tube methods (1,2). Lipemia following ingestion of cream, as well as intravenous infusion of cottonseed oil emulsion were found by one of us to result in significant shortening of whole blood clotting time in siliconized tubes and of plasma clotting time with Russell's Viper Venom(3). The question presented itself as to whether these *in vitro* findings reflected an increased tendency to clot formation *in vivo*. Therefore, a study was undertaken of the effects of

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* This study was supported by grant from Chicago Heart Assn.

intravenous administration of a fat emulsion[†] upon clotting of stagnant venous blood in the anesthetized dog, utilizing the experimental approach previously described by Wessler(4).

Material and methods. All experiments were performed on apparently healthy mongrel dogs weighing 25 to 48 lb, anesthetized with Pentobarbital. Sterile normal saline was infused into an antecubital or crural vein throughout the experiment. This infusion was briefly interrupted (2-3 minutes) only when the fat emulsion (10-40 ml) was injected through the same needle, later in the experimental procedure. Femoral and external jugular veins were widely exposed, and all demonstrable tributaries ligated and cut, thus freeing the exposed vessels from surrounding structures. All manipulations were carried out with greatest possible care so as to avoid injury to the vessel wall. Blood was trapped in these exposed veins by applying seraffine clamps at each end; additional clamps served to subdivide the vein into smaller segments, each containing about 1 ml of blood. At selected intervals, one small vein segment after another was excised; the contents were emptied into 0.1 M sodium citrate solution in Petri dishes and carefully inspected for the presence of a grossly visible clot. The *first* appearance of a fibrin clot, regardless of size, was taken as the end point. The interval from the moment of clamping to removal of the segment which contained the first noticeable clot was designated as *intravascular clotting time* (ICT). Blood samples were usually collected from antecubital vein or artery to coincide with clamping of veins on each side, as well as immediately preceding injection of fat emulsion and toward the end of the experiment, *i.e.*, after completing measurement of ICT on the second (usually the left) side (L in Fig. 1), whereupon the dog was sacrificed. Serum total fatty acids, analyzed in accordance with Bragdon's procedure (5), were utilized as index of the total lipid level. (Serum cholesterol and phospholipids were also measured but showed no significant changes incident to the experiments and are therefore not recorded.)

TABLE I. Intravascular Clotting Time (ICT) in 10 Fasting Dogs.

	Intravascular clotting time (min.)	
Dog No.	First side	Second side
1	40	L 40
2	L 30	30
3	"	L "
4	L "	"
5	L "	"
6	L "	"
7	L 40	>40
15	"	L 40
16*	"	L >4.0
18*	30	L 1.5

L denotes left side.

* Canine serum was inj. into peripheral vein immediately before measurement of ICT on left side. Vein segments were excised and inspected every 0.5-1 min. In dog 16, receiving 120 ml of serum for 20 min., no fibrin clot was seen in any of 5 available vein segments; in dog 18, receiving 90 ml within 2 min., a large clot was present in second segment inspected.

Results. Fasting values. In 15 fasting dogs (Tables I and II), ICT usually ranged between 30 and 40 minutes. Occasionally, no end point was reached, *i.e.*, no fibrin clot was seen in the last available vein segment (dogs 7, 20, 21, 23). There was remarkable consistency of results for the 2 sides of each dog (Table I).

Comparison of pre- and post-injection ICT values. In each of 5 fasting dogs (Table II), fat emulsion was injected rapidly after ICT had been measured in the jugular and femoral veins of the right side. Two minutes later, clamps were applied to the veins of the left side for estimation of ICT at time of marked lipemia. ICT was now 5 minutes shorter in dogs 8 and 9, which had received 10 ml of emulsion, and at least 10 to 15 minutes

TABLE II. Intravascular Clotting Time before and After Intravenous Injection of Fat Emulsion.

	Intravascular clotting time (min.)	
Dog No.	Before inj.	After inj.*
8	30	25
9	"	"
20	>30	20
21	"	"
23	"	15

* Left jugular and femoral veins were isolated by clamps 2 min. after emulsion had been inj. (10 ml to dogs 8 and 9, 40 ml to the others).

[†] Cottonseed Oil, 15.0; Dextrose, 4.4; Purified Soybean Phosphatide, 1.2; Water for Injection, q.s. ad 100 ml. This emulsion was furnished through courtesy of Don Baxter.

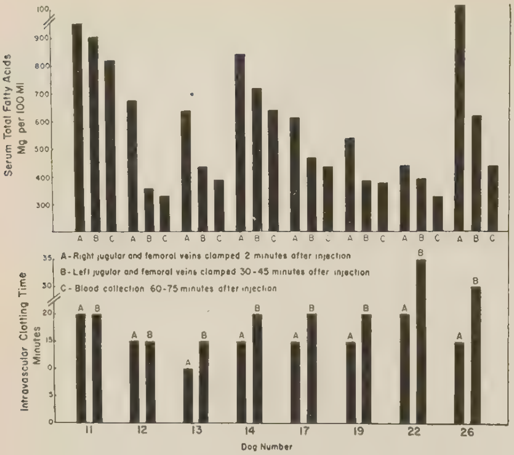

FIG. 1. Relation of intravascular clotting time to hyperlipemia after intrav. inj. of fat emulsion.

shorter in the other 3 dogs treated with 40 ml each.

Relation of ICT values to degree of induced hyperlipemia. In 8 dogs, ICT was determined in the veins of the right side 2 minutes after injection of the emulsion and 30 to 45 minutes later in the veins of the left side. In 6 of the 8 dogs, ICT was distinctly shorter at the peak of induced lipemia than at the time when this lipemia was clearly subsiding, indicating an inverse relationship between ICT and degree of lipemia in the respective dogs (Fig. 1). In all 13 dogs that received the fat emulsion, the immediate post-injection clotting times were below the lower limit of the fasting range, namely, less than 30 minutes (Table II and Fig. 1). This was true, with 2 exceptions (dogs 22 and 26), even of clotting times corresponding to the subsiding phase of lipemia (Fig. 1).

Discussion. The observed acceleration of clot formation in stagnant venous blood in dogs resulting from intravenous infusion of a fat emulsion correlates with the increased *in vitro* coagulability previously found in association with either intravenous or oral administration of fat in humans(3). Wessler(6) has drawn attention to the fact that reduced coagulability, as demonstrated by test tube methods, such as the one-stage (Quick) prothrombin time, does not necessarily mean decreased tendency to thrombosis. He found no delay in intravascular clot formation in dogs with severely reduced prothrombin activity following Bishydroxycoumarin medication. By the same token, shortening of whole blood clotting time or of "Stypven" clotting time cannot be interpreted necessarily as denoting an increased tendency to thrombosis. However, the data just presented *do* support the concept that lipemia results in an *in vivo hypercoagulable* state.

Rapid intravenous infusion of canine serum (not plasma!) into dogs was found by Wessler(7)—and verified by the present authors (Table I)—to cause clot formation in isolated veins in less than 2 minutes, as against 20 to 90 minutes in untreated dogs. His preliminary conclusion that this remarkable clot acceleration was due to active convertin (stable factor) in serum was subsequently disproved by him(6), and the mechanism of serum-induced venous thrombosis is not known. Lipemia-induced acceleration of clot formation, as observed in our experiments, is of much lesser degree but sufficiently distinct and consistent to be accorded potential clinical significance. Thus, when blood flow slows in a narrowed vessel, approaching conditions that exist in an isolated venous segment, namely, stagnation, thrombosis may be facilitated or precipitated by lipemia.

To be sure, this lipogenic clot acceleration may be specific for the fat emulsion used and attributable to certain of its ingredients. Among them, soybean phosphatide appears particularly suspicious since such substances have been shown to have platelet-like and other clot-promoting activity *in vitro*(2). Determination of the possible influence of physiologically induced, that is, alimentary lipemia upon ICT would be of much interest, particularly in view of recent reports describing myocardial and renal infarcts in rats on high-fat diets(8). In spite of the preliminary nature of the data presented, they would seem to suggest added caution in administering intravenous fat emulsions to human subjects, especially when a "temporary thrombotic" (local or systemic) state(9) may be suspected to exist or be impending.

Summary. Intravenous administration of a fat emulsion has been found to accelerate substantially the formation of a fibrin clot in isolated venous segments of anesthetized dogs.

This finding is interpreted as implying that induced lipemia may contribute to, or result in, an *in vivo* hypercoagulable state.

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Labeling of Intestinal and Lymph Cholesterol After Administration of Tracer Doses of Cholesterol-4-C^{14}.* (25002)

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In earlier studies(1,2) a tentative mechanism of cholesterol absorption was proposed in which it was postulated that transfer of cholesterol from lumen of intestine to lymph involves complete mixing with a pool of free cholesterol in the intestinal wall. Free cholesterol may be drawn from this pool for synthesis of lymph cholesterol esters and as a structural component of chylomicron. The data in those studies were obtained by feeding 40-44 mg cholesterol-4-C^{14} and determining changes taking place in both chemical and radioactive cholesterol fractions in the lumen, intestinal wall, and lymph. Under such conditions there was a marked increase in total lymph cholesterol in comparison with a fasting animal over 24 hour period, and of total cholesterol, 80-90% was esterified. Administration of 1-3 mg cholesterol-4-C^{14} dissolved in oil does not produce a chemical increase in total lymph cholesterol or change in percent esterified cholesterol over feeding of fat alone (3-5). Comparison of data obtained with administration of 40-44 mg cholesterol-4-C^{14} with that obtained by other workers(3-5) using 1-3 mg suggested that the latter amounts should be considered as *tracer* doses which labeled the endogenous cholesterol in the lumen and later the free cholesterol pool of intestinal wall. Thus, appearance of cholesterol-4-C^{14} in lymph appears to be a measure of reabsorption of endogenous cholesterol when fat alone is fed rather than a measure of exogenous cholesterol absorption. In this context, large doses of cholesterol-4-C^{14} employed in our earlier experiments(1,2) would be considered as *absorptive* doses since they increased pool size and turnover rate of free cholesterol in the mucosa and produced increases in levels of lymph cholesterol fractions. To test above suppositions and supply additional data on free cholesterol pool, the effect of feeding a small (tracer) dose of cholesterol-4-C^{14} on size and labeling of free and ester cholesterol pool of intestinal wall and of cholesterol fractions of lymph have been determined.

Methods and materials. Rats with thoracic lymph fistula were prepared and given saline to drink(1). Twenty-four hours after operation each animal received, by gastric intubation without anesthesia, 3 ml of aqueous emulsion containing 50 mg albumin, 150 mg glucose, and 3.27 mg cholesterol-4-C^{14} (0.5 μc) or one containing these constituents plus 292 mg oleic acid and 279 mg sodium taurocholate. Lymph was collected from 0-6 hours following administration of test meal and animals were sacrificed. The intestine was removed, washed, sectioned, and lipid extracts of intestine and lymph were prepared as de-

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TABLE I. Cholesterol and Cholesterol-4-C^{14} of Lymph and Intestine after Feeding Small Amount of Cholesterol-4-C^{14} to Lymph Fistula Rats.

	Cholesterol, mg						
Tissue†	Free	Free-C^{14}	Ester	Ester-C^{14}	Total	Total-C^{14}	Admin. cholesterol-4-C^{14} recovered, %
			Fasting‡				
Lymph	.79 ± .20*		1.42 ± .35		2.21 ± .19		
			Cholesterol-4-C^{14}				
Lymph	.74 ± .14	.02 ± .00	1.55 ± .30	.08 ± .01	2.29 ± .17	.10 ± .01	3.1 ± 1.0
Small intestine	12.55 ± 2.06	.34 ± .06	1.24 ± .28	.04 ± .00	13.79 ± 2.34	.38 ± .07	11.7 ± 2.8
			Cholesterol-4-C^{14} + oleic acid + sodium taurocholate				
Lymph	1.50 ± .42	.09 ± .02	3.00 ± .70	.30 ± .06	4.50 ± 1.12	.39 ± .08	11.9 ± 2.4
Small intestine	13.05 ± 2.26	.58 ± .14	1.24 ± .23	.09 ± .02	14.29 ± 2.49	.67 ± .16	20.5 ± 3.1

* Stand. error of mean.

† Values are avg of 5 lymph fistula rats killed at 6 hr after test meal. Cholesterol-4-C^{14} administered per rat was 3.27 mg (1 × 10^5 cpm/mg).

‡ Represents 6 hr fasting lymph sample from 4 rats.

scribed earlier(1,2). Free and total cholesterol of each extract were determined colorimetrically by the method of Sperry and Webb (6). C^{14}-activity of free and esterified cholesterol fractions of all lipid extracts were determined as previously described(1,2).

Results. (Table I). Administration of 3.27 mg cholesterol-4-C^{14} alone did not increase levels of lymph cholesterol fractions above those of fasting control. However, during the 6-hour period, 0.1 mg of labeled material was transferred to lymph and 80% of it was esterified. Addition of sodium taurocholate and oleic acid to cholesterol-4-C^{14} test meal produced a greater transfer of cholesterol-4-C^{14} to lymph (0.39 mg) and a significant increase in lymph cholesterol level. Levels of lymph free and esterified cholesterol fractions were approximately double those of the group fed cholesterol-4-C^{14} alone. The increase in total cholesterol of 2.21 mg (4.50-2.29 mg) was not accounted for by presence of fed cholesterol-4-C^{14} (0.29 mg). Thus, addition of oleic acid and sodium taurocholate to the test meal led to increase of approximately 2 mg of non-radioactive cholesterol in the lymph. Comparison of levels of free and esterified cholesterol in the intestinal wall in the 3 groups indicates that there were no significant increases in either free or esterified fractions upon administration of 3.27 mg cholesterol-4-C^{14} with or without oleic acid and sodium taurocholate. However, in the group which received cholesterol-4-C^{14} alone, 0.38 mg or 11.7% of amount fed, was present in the intestinal wall at end of 6 hours. Approximately 90% of this was present as free cholesterol-4-C^{14}. When sodium taurocholate and oleic acid were added to test meal there was a much greater uptake of cholesterol-4-C^{14} by the intestinal wall (0.67 mg *vs.* 0.38 mg).

Confirmatory information on free cholesterol pool of intestine, the relative endogenous dilution of both small and large doses of fed cholesterol-4-C^{14} were determined (Table II). When a small dose (3.27 mg) of cholesterol-4-C^{14} was fed, it was diluted to considerably greater extent in the intestine and lymph than a large dose (40 mg). Degree of this dilution was related to size of dosage fed. Since the small dose was one-tenth the large dose, it was diluted approximately 10 times in its transfer from lumen to lymph. It is thus suggestive that the relative effects obtained when large and small doses are fed

TABLE II. Endogenous Dilution of Small and Large Doses of Fed Cholesterol-4-C^{14}.

Cholesterol-4-C^{14} admin., mg*	Total cholesterol, cpm/mg Lumen	Total cholesterol, cpm/mg Lymph	Endogenous dilutions lumen cholesterol-4-C^{14}, No.
3.27	45200	8667	5.20
40	4348	2350	.54

* Values are avg of 5 lymph fistula rats killed at 6 hr after test meal. Test meals also contained 292 mg oleic acid and 279 mg sodium taurocholate.

are related to dosage fed, pool size, and turnover of pool.

Discussion. Our results indicate that feeding a small dose (3.27 mg) of cholesterol-4-C^{14} without sodium taurocholate and oleic acid did not influence size or turnover rate of cholesterol pool of intestinal wall or produce an increase in lymph cholesterol level. Addition of sodium taurocholate and oleic acid to test meal produced an increase in transfer of fed cholesterol-4-C^{14} to the lymph. However, the amount transferred did not account for the observed increase in lymph cholesterol level. This increased amount of cholesterol in lymph may have been derived from increased synthesis of cholesterol in the intestine. It was previously postulated(2) that during fat absorption an increase in cholesterol synthesis in the intestine would occur to support chylomicron and lipoprotein formation.

The data on cholesterol levels and specific activity of the cholesterol in lymph and intestinal wall after feeding small doses of cholesterol-4-C^{14} and the relative dilution of large and small doses are in agreement with the concept that cholesterol entering from the lumen is mixed with a pool of free cholesterol in the mucosa prior to incorporation into the chylomicron.

Summary. Small doses of fed cholesterol-4-C^{14} lead to labeling of cholesterol fractions of mucosa and lymph without increase in level or turnover rate of free cholesterol pool of the mucosa or in amount of cholesterol in lymph. Feeding tracer dose with sodium taurocholate and oleic acid increases the turnover rate of pool which leads to an increased amount of labeled and unlabeled cholesterol in lymph.

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Propagation of Duck Hepatitis Virus in Tissue Culture.* (25003)

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Virus-induced hepatitis has been described in man(1,2), duck(3-5), dog(6), and mouse (7-9), and agents of the latter 2 have been propagated in tissue culture(10-14). Much interest in comparative viral hepatitis stems from failure to propagate the virus(es) of human hepatitis in the laboratory(15). Since identification of this agent now depends on hazardous exploitation of man, alternate procedures with other host-specific hepatitis agents have been employed as possible prototypes. In this respect, some biological properties of duck hepatitis virus (DHV) have been studied.

Materials and methods. Assay and identification of DHV.† DHV-infected chick embryos were emulsified (10% w/v) in Hank's balanced salt solution (BSS). The preparation was partially clarified by centrifugation at 3,000 rpm for 20 minutes, ampulated, and stored at dry-ice temperature. Assays for DHV were performed by inoculation of 0.1 ml amounts of 10-fold dilutions of test fluids in phosphate buffered saline (pH 7.2) into

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† Kindly supplied by Dr. L. E. Hanson, Dept. of Veterinary Science, University of Illinois, Urbana.

7-day-old chick embryos *via* the chorio-allantoic cavity. Stock virus preparations usually killed the embryo in 2-4 days when diluted 10^6-fold. Infected embryos showed hemorrhagic lesions in skin and viscera. The lethal effect of DHV was neutralized by immune serum prepared in chickens and rabbits. Two intramuscular inoculations of virus in adjuvant (virus : mineral oil : arlacel; 0.5 ml : 1 ml : 0.1 ml, respectively) were given 2 weeks apart. Animals were exsanguinated 2 weeks later and sera were stored at –20°C. One ml aliquots of DHV pool were sealed in ampules and incubated at 37°C. At 5-day intervals, samples were assayed for virus content. Portions of virus pool were mixed with ⅓ volume of di-ethyl ether (Squibb) and stored overnight at 4°C. Mixtures were centrifuged at 2,000 rpm for 10 minutes, and aqueous fractions were assayed for virus content. Aliquots of virus pool were mixed twice with ½ volume of fluorocarbon (CCl_2F-$CClF_2$) (Genetron: General Chemicals, Inc., N. Y.) in a Virtis homogenizer as described by Gessler *et al.* (16). Aqueous portions were assayed for virus content. Seven-day-old chick embryos were washed in BSS and their bodies minced into approximately 1 mm pieces. Four to 6 washed explants were imbedded in chicken plasma onto the wall of each screw-cap tube. Nutrient fluid (NF) for tissue cultures contained BSS (90%), heat-inactivated chicken serum (10%), penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Occasionally Eagle's medium or solution 199 was substituted for BSS. The NF was decanted from proliferating explants after 48 hours incubation at 37°C in a roller drum apparatus. After addition of 0.1 ml of undiluted DHV pool and 0.9 ml of NF to each tube, they were reincubated. Cultures were examined daily, by low-power magnification, for evidence of CPE. At intervals, contents of several tubes were quick-frozen and thawed twice, pooled, and assayed for virus content. Subsequent virus passages involved addition of 0.1 ml of DHV tissue culture pool plus 0.9 ml of NF to tubes containing new chick embryo explants. At different passage levels, DHV was checked for identity by neutralization tests with immune serum. Some aspects of DHV replication in tissue explant cultures were studied by 1) assay of cultures at daily intervals following addition of DHV, 2) assay of NF from cultures renewed at 6-day intervals, 3) assay of cultures inoculated with 10-fold dilutions of DHV to determine minimal infective dose of virus, 4) assay of infected explants inoculated at various ages, and 5) assay of aging cultures in which the NF was not changed after the addition of DHV. Seven-day-old chick embryos were minced in BSS and treated repeatedly for 5 minutes with 0.25% Difco Trypsin 1:250 at 37°C. All resulting cells, except those from first treatment, were pooled, and washed at low-speed centrifugation. Approximately 100,000 cells in NF were transferred to each screw-cap tube. Cultures were incubated in stationary racks at 37°C for 3-6 days, then inoculated with DHV according to schedule described above with tissue explants. After 6 days at 37°C, cultures were quick-frozen and thawed twice, pooled, and assayed for virus. Pooled culture fluids were passaged (0.1 ml) through 5 or 6 series of trypsinized-cell cultures. Standard cell lines (HeLa, McCoy human fibroblast, and mouse "L" cell) and monkey kidney were propagated in stationary tubes at 37°C. Cells were loosened by agitation and/or by scraping from the tube-wall and transferred to tubes (approximately 100,000 cells/ml). Cultures were incubated in stationary racks for 5 days when confluent cell-sheets had formed and were inoculated with 10^6 LD_{50} of DHV. Subsequent examinations for CPE and for virus were similar to that described above with chick embryo explants.

Results. Properties of DHV. DHV is a relatively stable virus. It resisted treatment with ether and with fluorocarbon and survived for at least 3 weeks at 37°C (Fig. 1).

Propagation of DHV in tissue cultures. DHV has been propagated in chick embryo explants through 25 consecutive passages at 6-day intervals and production of virus particles exceeded amount in original inoculum (Table I). The cumulative time of DHV in tissue culture with sustained titer was 150 days which exceeded decay time of virus. Definite CPE was not observed; in fact, less virus was recovered from tissues which ap-

TABLE I. Propagation of DHV on Explanted and Trypsinized Chick Embryo Cultures. Passaged at 6-day intervals.

Tissue cultures	Passage No. and virus titer								
	Base	1	2	3	4	5	15	20	25
Trypsinized	10^{-6}	10^{-4}	10^{-3}	10^{-1}	Neg.	Neg.			
Explanted	10^{-7}	NT*	NT	NT	10^{-4}	10^{-5}	10^{-3}	$10^{-4.5}$	$10^{-3.5}$

* Not tested.

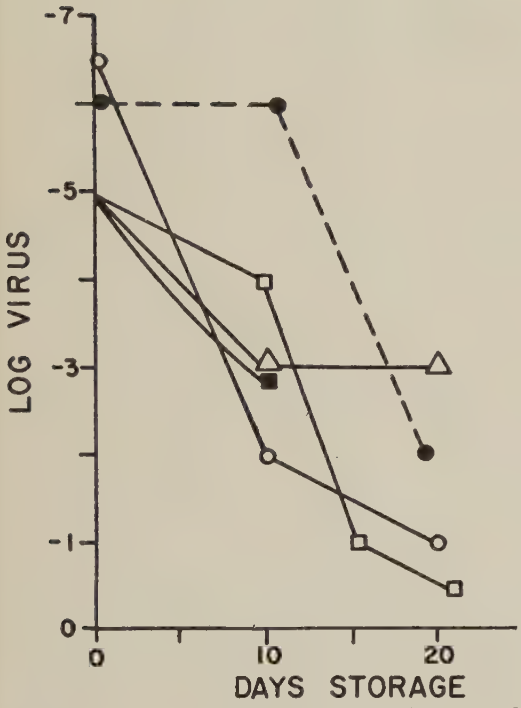

FIG. 1. Survival of duck hepatitis virus stored without tissue at 37°C. Each line represents a separate test series.

peared degenerated than from actively growing tissues. LD_{50} titers of DHV from individual tissue culture passages ranged 10^{-3} to 10^{-5}. Tissue explants were infected by as little as 10 LD_{50} of DHV. Substitution of Eagle's medium or solution 199 for BSS did not influence virus production significantly. DHV multiplied in tissues which had been explanted 4-21 days previously. When NF of infected cultures was replaced 4 times at 6-day intervals, DHV reappeared in the NF with LD_{50} titers between 10^{-2} and 10^{-3}. When NF of infected cultures was not changed for 32 days, DHV was recovered in dilutions of 10^{-4}. Initial titer of virus in NF (10^{-5}) declined to LD_{50} 10^{-1} on first day after inoculation and climbed gradually to sustained titer of LD_{50} 10^{-4} after 4 days.

Duck hepatitis virus was recovered from first 3-4 passages in trypsinized chick embryo and disappeared thereafter (Table I). Virus assays on consecutive trypsinized-cultures simulated the normal decay curve (Fig. 1) and in some instances this decay was accelerated. DHV disappeared more rapidly in cultures of established cell lines than in cell-free medium alone. CPE was not noted in inoculated cell lines.

Discussion. Failure to detect propagation of DHV in mammalian cells is not surprising in view of its restricted spectrum of infectivity. However, failure of DHV to propagate in trypsin-treated chick embryo cells is more difficult to explain: trypsinization may have destroyed either a substance essential to virus replication or individual cells in which DHV reproduced selectively. As a check on technic, influenza A virus was passaged successfully at 72 hour intervals through 6 series of similarly prepared trypsinized chick embryo cells. This emphasizes the unusual relationship of DHV to trypsin-treated cells.

DHV has been propagated in chick embryo explant tissues without CPE. Absence of definite CPE in tissue explants infected with mouse hepatitis virus(12-14) and in tissue cells presumably infected with human hepatitis virus(17) indicates the need for other procedures of virus detection. Addition of an essential nutrient to the culture fluid may elicit a CPE, as was done with measles virus (18).

Summary. Duck hepatitis virus (DHV) survived storage for at least 21 days at 37°C. It survived treatment with di-ethyl ether and with fluorocarbon. DHV has been propagated through 25 consecutive passages in tissue cultures prepared with chick embryo explants. It was not propagated in cultures of trypsinized chick embryo cells or in cell lines of mammalian origin.

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Electroencephalographic Evidence of Osmosensitive Elements in Olfactory Bulb of Dog Brain.* (25004)

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Verney and his associates(1,2) presented physiological evidence of the presence, within the bed of internal carotid artery of the dog brain, of elements sensitive to changes in osmotic pressure of blood. Activation of these "osmoreceptors" by hypertonic solutions stimulated release of neurohypophysial antidiuretic hormone (ADH). The precise location of the receptors was unknown, though it was suggested that they might lie within the supraoptic nucleus of the hypothalamus. Studies which employed electroencephalographic (EEG) recording technics in rabbits and in cats(3,4,5,6) have indicated that osmosensitive elements may lie rostral to the hypothalamus in the olfactory system. Characteristic EEG changes were observed in olfactory projection areas following intracarotid injection of hypertonic saline which activated release of oxytocin(4) as well as ADH(5). In a related investigation(6) hypertonic solutions were shown to act independently on hind brain to induce consistent changes in arterial pressure. The vast amount of information assembled by Verney *et al.* in the dog made it desirable to investigate osmoreceptor function in this species with electrophysiological methods.

Methods. A total of 24 mongrel dogs of both sexes were prepared under ether or sodium pentothal (20 mg/kg) anesthesia. Subsequently only a local anesthetic (4% procaine) was employed while animals were immobilized with gallamine triethiodide (Flaxedil) administered intravenously, and maintained with respirator pump. Blood pressure was recorded from femoral artery with a Statham transducer and a Varian inkwriter. Intracarotid injections (1-5 ml) of the following solutions were given through a polyethylene cannula: 0.15 M NaCl (isotonic), 1-1.5 M NaCl (hypertonic), 2 M sucrose and glucose. The scalp was incised and a skull flap removed. Using a stereotaxic instrument

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[†] Fellow of Commonwealth Fund, 1957-59.

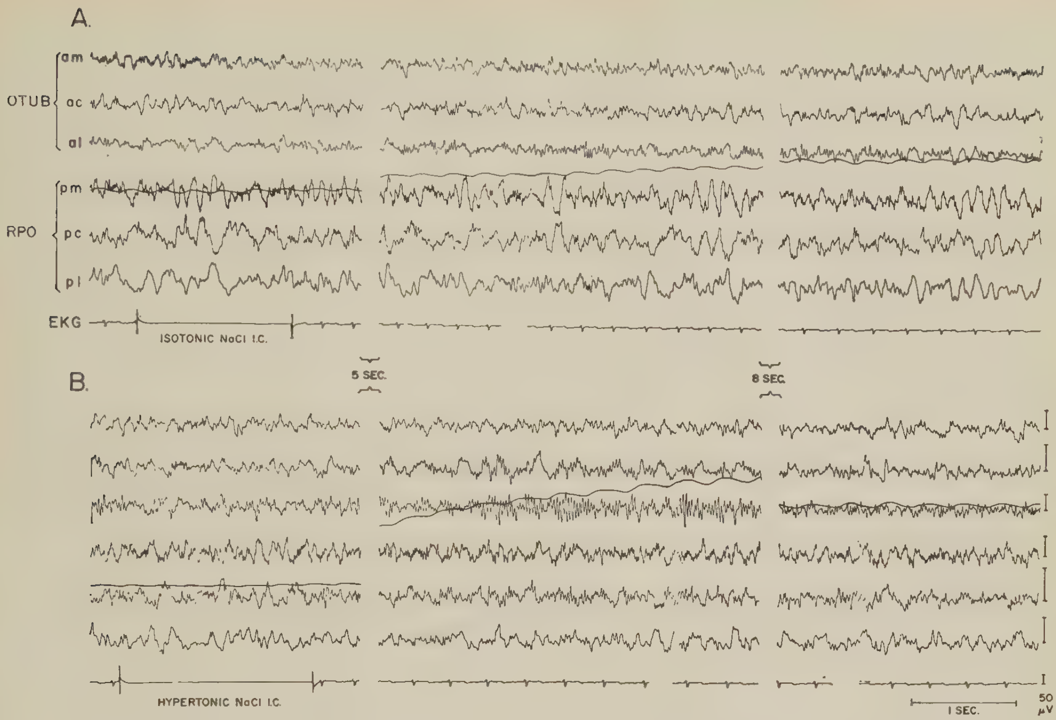

FIG. 1. Typical EEG response to intracarotid hypertonic saline. A. Control record after inj. of isotonic saline (3 ml 0.15 M NaCl). B. EEG response to hypertonic saline (3 ml 1.0 M NaCl). Arterial pressure is indicated by line running through EEG records. In both cases it starts from base line of 70 mm Hg and after hypertonic saline it reaches a value of 130 mm Hg. Heart rate is indicated by electrocardiogram (EKG). Small letters inside brackets correspond to electrodes from which EEG tracings were taken. The first 3 channels reflect activity in olfactory tubercle (OTUB) recorded from bipolar electrodes 3 mm apart and in the same transverse plane; the most lateral (al) is just medial to lateral olfactory tract; the central (ac) is in anterior commissure; most medial (am) is in nucleus accumbens. In a similar manner, a row of 3 electrodes is placed 3 mm posteriorly in the pre-optic region (RPO); the EEG tracings are thus labeled correspondingly from medial to lateral (pm, pc, pl).

patterned after that of Hume and Ganong (7), a movable bracket of 6 concentric bipolar electrodes, oriented by use of x-rays, was lowered into deep brain structures. Electroencephalographic recordings were made with use of 8-channel Grass inkwriter. In 2 cases the response to hypertonic saline was also monitored oscilloscopically.

Results. Intracarotid injections of hypertonic saline in the dog induced characteristic EEG changes (Fig. 1). Control injections of isotonic saline (A) caused no appreciable alterations in either electrical activity of brain or blood pressure. The latter was recorded to serve as indication that hypertonic saline was reaching the brain(6). The typical EEG response to hypertonic saline (Fig. 1, B) is most marked in channel 3. In general, 5 to 8 seconds after intracarotid injection, a high amplitude burst of synchronous activity appears and lasts for approximately 5 seconds. Its frequency lies in the 40-70 cycles/sec range and recorded with the use of oscilloscope as well as electroencephalograph.

The most dramatic response to hypertonic saline (Fig. 1, B, channel 3) is discretely localized just medial to lateral olfactory tract. It is also present to a lesser degree in the anterior commissure (channel 2). In 18 animals it has been recorded from the lateral part of olfactory tubercle, including lateral olfactory tract, and extending anteriorly into the olfactory peduncle. Only a relatively slight change of a synchronous nature can be recognized in the preoptic region (Fig. 1, B, channel 5). When seen at this antero-postero level (3 out

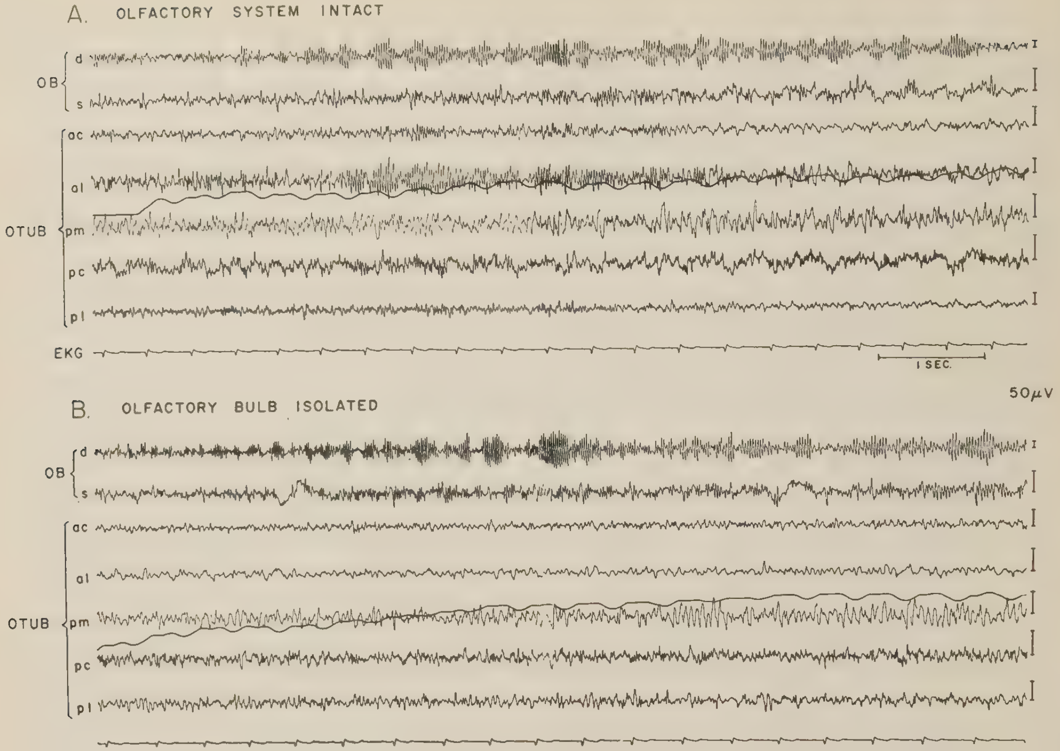

FIG. 2. EEG responses in olfactory bulb before and after isolation from basal forebrain. Both EEG records begin 3 sec. after intracarotid inj. of hypertonic saline (1.5 ml, 1.5 M NaCl). A. Synchronous response appears in both OTUB and OB before sectioning between them. B. Synchronous response appears only in OB after isolating it from rest of brain. In both cases arterial pressure increased approximately 50 mm Hg from 120 mm Hg baseline. EEG trace in channel 1 is taken from electrode (d) in the olfactory bulb (OB) whose tip rests in mitral cell layer; another (s) rests on bulb's surface. Two additional rows of bipolar electrodes are placed deep in the olfactory tubercle (OTUB). The anterior lateral electrode (al) is in the lateral olfactory tract and another (ac) is placed 3 mm medial to this at same depth. The other row is 2 mm posterior in region of diagonal band. Three electrodes are positioned 3 mm apart; the most lateral (pl) lies just medial to lateral olfactory tract; the most medial (pm) and the one in between (pc) are at same depth as lateral electrode.

of 9 animals whose anterior electrodes were showing positive changes), the response has been located in the lateral preoptic area not far from the nucleus of lateral olfactory tract.

Since the induced activity appeared to be stronger in the more anterior regions, additional electrodes were placed in the olfactory bulbs (OB) of 5 dogs. Typical EEG changes in response to hypertonic saline have been observed in the OB in each of these cases (Fig. 2, A). The OB record in channel 1 shows the high amplitude synchrony, which also appears in the olfactory tubercle, quite marked in channel 4. Other areas close by can be seen to pick up this change of activity only to a slight degree. To determine from which of these regions the activity was emanating, a complete section between the bulb and the tubercle was made with a blunt instrument. Following this the response to hypertonic saline was no longer obtained in the OTUB, while the OB was still reactive (Fig. 2, B). In addition, the activity induced in the olfactory bulb is independent of the olfactory epithelium: a procaine spray blown into the nostril, which will block the bulb's response to a blast of odor-laden air, does not interrupt the response to hypertonic saline. The activity, therefore, has characteristics of Adrian's "intrinsic olfactory activity"(8).

These responses are not dependent on specific receptors for sodium or chloride, but rather on osmosensitive elements, since they can be evoked by hypertonic glucose and hypertonic sucrose. Whatever influence they may have on the activation of the supraopticohypophysial system is a question for further study.

Conclusion. Intracarotid injections of hypertonic saline and sugar solutions in the dog induce characteristic synchronous EEG changes in the olfactory bulb which are independent of influences from the olfactory epithelium or the rest of brain. In the intact brain, these osmosensitive elements in the bulb transmit their nervous impulses caudally along olfactory pathways, but their influence on the supraoptico-neurohypophysial system is unknown.

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Infectivity of Ribonucleic Acid (RNA) from Type I Poliovirus in Embryonated Egg.* (25005)

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Multiplication of MEFI strain of Type II poliovirus in the chick embryo after prolonged propagation in the suckling hamster is now well established(1,2,3,4). No reports of confirmation of reproduction of Type I or III poliovirus in chick embryo have appeared (see (5)). However, it has been reported(6) that *in vitro* cell cultures from chorio-allantoic membrane of chick embryo can support 10 to 20 transfers of all 3 types of poliovirus, to an extent indicative of multiplication. The present report describes synthesis of Type I poliovirus in embryonated eggs after inoculation by amniotic route with ribonucleic acid (RNA) derived from Type I (Mahoney) poliovirus. Polio-RNA previously exposed to ribonuclease (RNAase) failed to yield the plaque-forming agent.

Materials and methods. Materials and procedures leading to this study have been published(8); additional or modified technics will be described. Mahoney Type I poliovirus grown on human amnion cell culture was obtained from Cutter Labs. (Berkeley, Cal.) as 100-fold concentrate designated E_1. Ribonucleic acid (RNA) preparations were made by phenol method of Gierer and Schramm(9), which consists of 3 extractions with phenol, followed by removal of phenol by ether extraction and subsequent removal of the ether by nitrogen. For our preparations, freshly distilled phenol was used; pH of preparation was adjusted finally by addition of a tenth volume of 0.9 M NaCl containing 0.04 M sodium phosphate at pH 7.6. Aliquots were used which, without additional dilution, had been frozen in a CO_2 box at −70°C, and thawed once. Occasionally fresh preparations were used. The plaque forming capacity of polio-RNA was usually tested simultaneously with experimental test fluids. Most viral assay tests were carried out in monolayers of a line of human amnion cells(10), although these were supplemented occasionally with small tests using monolayers of HeLa L5, a clonal line used previously(8). When comparative tests were done, no significant dif-

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ference in sensitivity of cell lines was observed. Monolayers grown in an atmosphere of 5% CO_2 in air at 35°C, usually for 2 days, were washed with Hanks' solution minus Ca, Mg and PO_4 ions ("simple Hanks' solution"). The same solution was used for second washing when inoculum was tested for presence of whole virus. However, a second washing of alkaline, hypertonic salt solution was used when detection of infectious RNA was sought. The alkaline, hypertonic solution consisted of 0.65 M NaCl containing 0.03 M tris (Sigma 7 to 9 buffer grade (hydroxymethyl aminomethane)) at final pH 7.6. The washing solution was removed as completely as possible(11). The monolayers were inoculated with test fluids, followed after half an hour at room temperature with an overlay of agar; and, after further incubation in 5% CO_2 in air, for 2 or 3 days at 35°C, by a second overlay of agar containing neutral red. Three or 4 hours later, and again the following day, plaques were counted. In the Tables plaque-forming units (PFU)/0.1 ml inoculum are reported. Crystalline ribonuclease (RNAase) of bovine origin was obtained from Armour Co., Chicago. *Antisera and globulin.* Pools of serum from rhesus monkeys, before ("normal") and after hyperimmunization by repeated intramuscular injection of monkey spinal cord and medulla infected with Type I or II poliovirus emulsified in adjuvants served as source of globulins. Globulins were thrice precipitated in half-saturated ammonium sulfate solution, alternately dissolved in distilled water, and finally dialyzed against Earle's solution (BSS) (12).

Fertile eggs, usually 7 days of age, were obtained from Shamrock Farms, Milltown, N.J. After arrival in laboratory they were incubated at 36°C. The inoculum from a syringe with 22-gauge needle was placed into the amniotic sac. To insure proper location of needle, some fluid was drawn up. When crystal clear amniotic fluid was seen in neck of syringe, the inoculum was injected. *Results. Choice of route of inoculation.* Since a prime characteristic of polio-RNA(7,8) is its inactivation by low concentrations of RNAase, an enzyme ubiquitous in animal cells, it was essential to find a route of inoculation such that RNA would come into contact with susceptible cells before its destruction by RNAase. To determine relative desirability of various routes, fluids from "normal" embryonated eggs, 8 to 10 days of age, were compared with a physiological (Hanks') solution for their inactivating effect on polio-RNA. In one test, amniotic fluids from 2 embryos (8 and 9 days old) were compared with 2 sets of controls consisting of Hanks' solution at pH 7.4. One part of each fluid, mixed with 9 parts of 1/50 dilution of RNA, stood 5 minutes at room temperature before inoculation of HeLa L5 monolayers. From 5 plates each the average number of plaques formed in presence of amniotic fluids was 148 and 164 compared with control averages of 109 and 162. In another test 3 amniotic fluids from 9-day-old eggs yielded plaque counts of 85, 80+, 100+, compared to controls with 0.15 M NaCl at pH 8.0, which yielded 65 and 90+. Thus no inactivating effect of amniotic fluid on polio-RNA was observed. On the other hand, when a few yolks and allantoic fluids were tested for their effect on plaque-forming capacity of polio-RNA, some reduction in infectivity was observed. Thus, since amniotic fluids gave no indication of an inactivating effect on RNA in a controlled *in vitro* system, the amniotic route was chosen as most favorable for inoculation of RNA.

A test was done to determine whether amniotic fluid was devoid also of DNAase (desoxyribonuclease) activity. We are indebted to Miss Grace Leidy for testing effect of amniotic fluid from a pool of four 9-day-old developing eggs on the transforming principle (TPd_{SM}) in an *H. influenzae* system using Rd cell culture. No DNAase activity was demonstrable in the amniotic fluid pool under conditions which would detect as little as 1 μg/ml of the enzyme. Thus the amniotic route of inoculation may prove valuable for study of both types of nucleic acid, DNA (desoxyribonucleic acid) as well as RNA, from viruses to which the chick embryo is not susceptible in the usual sense.

Effect of fluids from embryonated egg on whole poliovirus. Chick embryo suspension and fluids from embryonated eggs were tested for their effect on plaque-forming capacity of E_1 poliovirus (a sample of that from which the polio-RNA had been derived). In 3 tests, there was no inactivating effect on whole Type I poliovirus by 5 to 30 minutes' exposure to 50% chick embryo suspension, 10% yolk, undiluted amniotic or allantoic fluid, as compared with Hanks' solution, under conditions known to favor neutralization by specific antibody. This result is consistent with the finding in many laboratories that Type I poliovirus may survive for long periods in the embryonated egg, although multiplication has not been confirmed. Thus, the failure to multiply is not attributable to any demonstrable neutralizing effect of tissues or fluids of the egg.

Result of inoculation of embryonated eggs with polio-RNA. Fertile eggs of 7 to 8 days' incubation were inoculated with 0.1 ml "undiluted" polio-RNA by the amniotic route. This dose, as assayed on monolayers, varied in different preparations from 300 to 4000 PFU tested in most cases simultaneously with materials recovered from the egg. Higher assays would be expected under optimal test conditions now known(11). Inoculated eggs were replaced in the incubator and at intervals from ½ hour to several days, a few eggs were removed for samples of amniotic fluids and tissues which were frozen once before assay. Each embryo, and each pair of allantoic and amniotic sacs, was ground in glass homogenizer with enough Hanks' solution to make a 50% suspension. 0.1 ml of each suspension or undiluted fluid was tested for plaque-forming capacity on a HeLa L5 monolayer. Results of 7 experiments are summarized in Table I. After incubation for ½ to 2 hours, no plaque-forming agent was recovered from embryos, sacs or amniotic fluids. At 4 hours, about half of the eggs, and at 6-8 hours nearly all eggs yielded plaque-forming agent. It was demonstrated earlier in significant amounts in the embryo and amniotic sac than in amniotic fluid, although the number of plaques observed varied considerably. After 20-24 hours' incubation plaque-forming agent was recovered from a minority of tissues and fluids, and thereafter, at 2 to 7 days, little or none.

Assays in which the alimentary tract with viscera was compared with remainder of embryo failed to yield any significant difference.

Emergence of a considerable amount of the plaque-forming agent earlier and in a higher proportion in tissues (embryo and amniotic sac) than in amniotic fluid may reflect intracellular formation of plaque-forming agent with subsequent release into the amniotic fluid. The time of virus demonstration, with maximum positive results at 6-8 hours, is compatible with growth and release of poliovirus observed in single cells by Lwoff *et al.*(13).

Our earliest demonstration of plaque-forming agent, *viz.*, 4 hours, may indicate a more rapid synthesis of virus from RNA than from intact virus, as compared to Lwoff's experiment. However, further investigation under comparable conditions is necessary to establish this comparison. Thus inability of Type I poliovirus to propagate in the developing chick as observed by several investigators may be due not to inability of cells to synthesize whole virus, when the active nucleic acid gains intracellular position, but rather to failure of absorption or entrance.

Nature of incitant to virus synthesis. To test the possibility that the plaque-forming agent produced in embryonated eggs after inoculation of polio-RNA might conceivably represent survival in the RNA preparation of a trace of residual intact virus present, RNA was exposed to RNAase (final concentration 15 μg/ml) before inoculation of eggs by the amniotic route. This experiment was controlled by an equal number of eggs which received RNA alone. RNA (with or without RNAase, added in dry form) stood 5 minutes at room temperature before inoculation into each of 2 groups of 6 eggs. Amount of "undiluted" RNA inoculated into each egg represented 900 PFU. At 10 minute intervals, from 30 to 80 min, an egg inoculated with polio-RNA alone was opened. The embryo, allantoic and amniotic sacs, and amniotic fluid were removed. No plaque-forming agent was demonstrable from 6 embryos and sacs or 5 amniotic fluids tested by inoculation onto human amnion cell monolayers washed with

TABLE I. Infectivity of Tissues and Fluids Recovered from Embryonated Eggs at Various Intervals after Inoculation of Polio-RNA by the Amniotic Route.

Eggs 7-8 days old at inoc. Incubation period	Material from inoc. egg Suspension (50%) or fluid (undil.)	Test No.† I	II	III‡	IV	V‡	VI	VII	Summary Total No. positive / Total No. tested Emb. and /or sacs	Amniotic fluid
		No. of plaques on each plate								
30-80 min.	Emb. and/or sacs	0,0,0,0,0,0							0/6	
	Amniotic fluid	0,0,0,0,0								0/5
2 hr	*Idem**			0,0,0,0	0,0,0				0/7	
				0,0,0,0	0,0,0					0/7
4 "	"			0,0	12,0,2	7,Cf,0,10,0,45			6/11	
				0,0	0,0,0	0,0,3				1/8
6 "	"		Cf,1,Cf		27,81,36	0,64,Cf,44,35,17			11/12	
			83,0,5		1,3,1	0,0,0,5,0,0				6/12
8 "	"	191,Cf,110,148,177,82			3,0,0,0	Cf,52,Cf,57,23			12/15	
					1,0,0,0	Cf,3,Cf,0,0				4/9
18-24 hr	"		2,0,0	8,0,16,12,75,0,0,0		4,0,0,0,0,0	156,53	0,0,0,0,0,0,0,0	4/20	
			7,1,0			0,0,0,0,0,0	89,Cf	0,0,0,0,0,0,0,0		8/28
2 days	"		0,0,0						0/3	
			23,0,0							1/3
5-7 days	"		0,0,0				1,0		1/5	
			0,0,0				3			1/4

Test procedure: 0.1 ml each fluid was inoculated onto monolayer of human amnion cells.

* As in the first (30-80 min. incubation) group, the top line indicates Emb. and/or sacs, and the bottom line Amniotic fluid throughout.

† Plaque-forming capacity of materials in each test was assayed simultaneously, after single cycle of freezing and thawing followed by centrifugation.

‡ In tests III and V, all test fluids were exposed to RNAase (at final conc. of 10 μg/ml) for 5 min. at room temperature before inoculation. Thus plaque formation was interpreted as caused by intact virus and not by infectious RNA. RNA preparations to inoculate eggs proved infectious when tested on 5 occasions simultaneously with materials removed from eggs.

alkaline, hypertonic salt solution. However, after 8 hours, all embryos and sacs of 6 eggs which received RNA alone yielded plaque-forming agent with counts/plate of 191, 250, 110, 148, 177 and 82. In contrast, comparable materials recovered from 6 eggs inoculated with RNA, and exposed to RNAase immediately before inoculation, yielded, after a similar 8-hour period, no plaque-forming agent. The outcome of this experiment makes it highly improbable that plaque-forming agent recovered from the egg originated from a trace of active virus remaining in the nucleic acid preparation. On the contrary, the experiment demonstrated that the inciting agent proved susceptible to action of RNAase, a cardinal property of the infectious nucleic acid.

Identification of plaque-forming agent formed in embryonated egg. The effect of RNAase and of anti-I globulin on plaque-forming capacity of a pool of embryos and sacs from eggs harvested 8 hours after inoculation with RNA alone was measured. The plaque-forming agent recovered from eggs yielded in one test essentially the same number of plaques after exposure for ½ hour at room temperature to RNAase (169 and 125) as to BSS (141, 182, and 119) but none after exposure to anti-I globulin, thus fulfilling requirements for identification as Type I poliovirus. The result was confirmed in 3 other tests. The type-specificity of the plaque forming agent, enhanced in titer by passage through human amnion cell culture, was determined. The freshly harvested virus was titrated in presence of 1/10 dilutions of monkey sera. In presence of "normal" serum, and of anti-II serum, 7.3×10^7 and 5.5×10^7 PFU/ml, respectively, whereas in anti-I serum, 5.3×10^2 PFU/ml were observed, thus demonstrating type-specificity of poliovirus recovered from eggs, after human amnion cell passage.

Egg passage of plaque-forming agent. To determine whether Type I poliovirus synthesized in eggs in response to inoculation with polio-RNA might multiply on passage through embryonated eggs, Type I virus recovered 8 hours after inoculation of eggs with polio-RNA was passed by the amniotic route. A suspension of embryos and sacs, containing 160 plaque-forming units/0.1 ml, was inoculated into eggs. Embryos and sacs harvested 8 hours later yielded almost no virus, while a pool of allantoic and amniotic fluids yielded an average of 30 plaques/0.1 ml, suggesting survival of some of the inoculated virus. In 2 experiments, eggs harvested at 18-24 hours yielded no plaques from tissues or fluids.

The possibility that larger doses of poliovirus recovered from eggs inoculated with polio-RNA might be more effective in passage led to attempts with virus enhanced in titer by passage through human amnion cell culture. Such virus was inoculated into eggs in doses from $10^{4.8}$ to $10^{6.8}$ PFU. After 6 days(4), 1/100 to 1/1000 of amount inoculated was recoverable. On second passage, only a few plaque-forming units were demonstrable, and none on third passage. Virus survival in the egg was comparable to that of Type I E_1 poliovirus (passaged in parallel) which had had no previous contact with the egg. Thus there was no indication that the egg-derived virus could multiply in the embryonated egg. Whether virus recovered from eggs could be adapted on egg passage has not been determined. Virus recovered from eggs has been kept in frozen state for future studies.

Summary. 1) Phenol extraction of Type I poliovirus yielded a plaque-forming agent which is presumably ribonucleic acid (RNA) because it is inactivated by RNAase. Such ribonucleic acid derived from Type I (Mahoney) poliovirus induces formation of plaque-forming agent in the embryonated egg, inoculated by amniotic route. The plaque-forming agent recovered from embryo tissue, sacs, and fluids has been identified as type specific, Type I poliovirus. Highest yields occurred at 6 to 8 hours after inoculation of eggs with polio-RNA. 2) Polio-RNA exposed to RNAase immediately before inoculation into embryonated eggs failed to incite formation of plaque-forming agent. This establishes that it is RNA itself, and not some undetectable trace of intact virus, which incites production of whole virus in the egg. 3) Propagation of virus recovered from eggs by further

passage in embryonated egg have not succeeded. 4) This demonstration of production of Type I poliovirus from nucleic acid in the embryonated egg, without subsequent multiplication, provides a system for biochemical study of genesis of whole virus from its nucleic acid. An advantage of the system is that the inciting agent (infectious RNA) can be differentiated completely, by its susceptibility to the action of RNAase, from whole virus progeny.

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Antagonism of Succinylcholine to Chlorpropamide Induced Hypoglycemia. (25006)

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Hypoglycemic activity of 1 (p-chlorobenzenesulfonyl) 3-N propylurea (chlorpropamide, Diabinese®) has been well established experimentally(1) and clinically(2). Following high doses of chlorpropamide in animals, a decrease of spontaneous motor activity and drowsiness were noted. This prompted an electroencephalographic study in the course of which cats were immobilized with succinylcholine. Surprisingly enough, severe hyperglycemia was found and normally effective doses of chlorpropamide did not lower blood glucose. This study was designed to elucidate this antagonistic action.

Methods. Forty-five cats and 100 rats were employed. Cats of both sexes ranging in weight from 2.5 to 3.5 kg were fasted 16-18 hr. Anesthesia was induced by intraperitoneal injection of pentobarbital sodium 40 mg/kg. A tracheal cannula was inserted and femoral vein was cannulated for injection of various agents and collection of blood samples. Whenever necessary, artificial respiration was maintained by Palmer Pump using 20 to 30 cc of air. Bilateral adrenalectomy was performed by lumbodorsal approach. Such animals were maintained 24 hr by subcutaneous injection of 1 mg/kg of cortisone. Male albino Wistar rats weighing 275-350 g were used necessitating artificial respiration. A tracheal cannula was inserted and attached to a Harvard Pump. For all other experiments, rats weighing 140-175 g were used. Bilateral adrenalectomy was performed 2 days prior to testing; animals were maintained by giving 1% saline in drinking water. Partial hepatectomy was performed under ether anesthesia 1 day prior to testing with removal of median and left lobes which comprise about 70% of liver tissue(3). All rats were fasted 18 hrs. The sodium salt of chlorpropamide was administered intravenously. Other agents were given as specified. Blood glucose determinations were performed with Auto Ana-

TABLE I. Effect of Chlorpropamide on Blood Glucose.

	No. of animals	Chlorpropamide dose, mg/kg I.V.	Blood glucose, mg %							
			0′	45′	60′	90′	120′	180′	240′	300′
Anesthetized* cats	4	25	70.0 ±7.26†		70.4 ±20.40		62.1 ±21.00	72.5 ±21.50	76.0 ±22.80	73.5 ±23.10
	3	50	84.0 ±18.16		52.0 ±9.84		34.0 ±4.18	29.0 ±3.51	30.0 ±6.00	33.0 ±4.35
	3	100	73.0 ±5.83		44.0 ±7.57		34.0 ±3.51	30.0 ±3.78	38.0 ±6.34	40.0 ±7.17
Unanesthetized cats	3	25	69.0 ±6.92		46.0 ±13.00		37.0 ±7.00	39.0 ±6.63	44.0 ±9.27	59.0 ±2.08
	3	50	60.0 ±5.56		46.0 ±6.85		41.0 ±5.78	37.0 ±8.12	31.5 ±7.87	36.0 ±6.48
	3	100	65.0 ±7.76		38.0 ±2.00		34.0 ±2.51	28.0 ±1.73	31.0 ±4.41	36.0 ±2.86
Anesthetized* rats	6	25	74.4 ±3.04	66.4 ±2.06		54.6 ±1.95			62.6 ± .89	
	6	50	81.9 ±1.93	68.0 ±1.61		44.6 ±1.33			57.5 ±2.53	
	6	100	81.4 ± .29	56.7 ±1.34		42.1 ±6.40			53.0 ± .29	
Unanesthetized rats	6	25	86.7 ± .28	82.8 ±4.01		67.6 ± .29			75.7 ±2.81	
	6	50	89.7 ±1.87	63.1 ±2.42		55.3 ±1.83			62.0 ± .60	
	6	100	79.0 ±2.18	51.0 ± .23		45.8 ±1.22			48.7 ±1.60	

* Anesthetized with pentobarbital sodium, 40 mg/kg I.P.
† ± S.E.

lyzer, employing modification of Hoffman's (4) micro method.

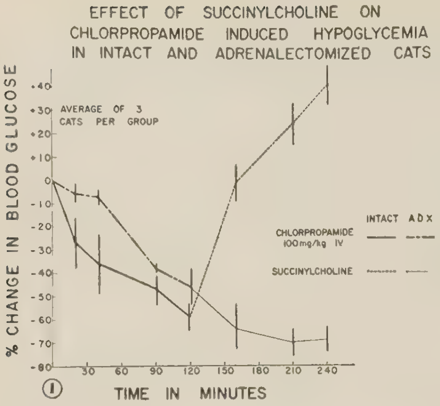
Results. Hypoglycemic activity of intravenously administered chlorpropamide in anesthetized and unanesthetized cats and rats was studied. A definite hypoglycemic response occurred in both species (Table I). To insure maximal hypoglycemic responses in anesthetized, and unanesthetized animals, chlorpropamide was used in subsequent experiments at 100 mg/kg.

Succinylcholine at doses sufficient for complete muscular paralysis (5-8 mg/kg in rat and cat) was administered intravenously. The dose at which respiration ceases is between 1-2 mg/kg. Supplementary doses were given as necessary. Succinylcholine in the intact cat caused a complete reversal of hypoglycemic response to chlorpropamide (Fig. 1). Similar results were obtained in the rat. However, in the adrenalectomized cat, no antagonism of blood glucose was observed (Fig. 1). Because of extreme sensitivity of the adrenalectomized rat to large doses of chlorpropamide, a similar experiment was not carried out in the rat. Death from hypoglycemic shock resulted during chlorpropamide pretreatment or immediately following succinylcholine.

Since succinylcholine appeared to alter the hypoglycemic response obtained with chlorpropamide, another immobilizing agent was investigated. Curare, injected intravenously to cats in doses from 3-5 mg/kg, also reversed blood glucose lowering action of chlorpropamide. Following initial reversal of the hypoglycemic response with chlorpropamide, blood glucose levels gradually declined for duration of the 2 hr period.

Six rats were hepatectomized, given chlorpropamide, and after 1 hr immobilized with succinylcholine. Blood glucose values showed continuous decline in spite of administration of succinylcholine (Table II).

Since ether elevates blood glucose through liberation of adrenal medullary hormones



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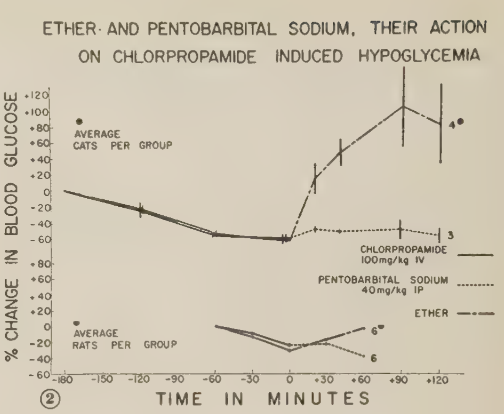

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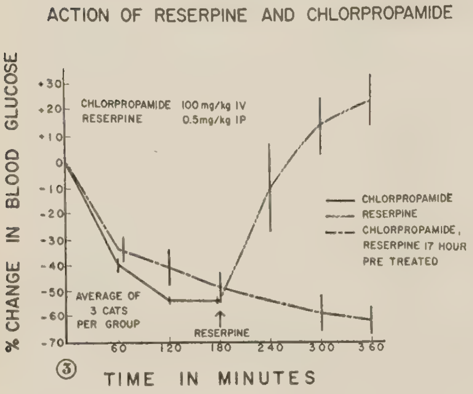

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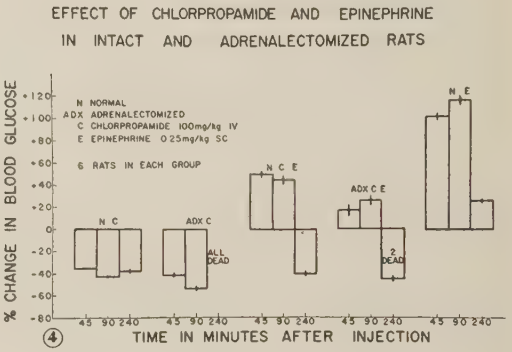

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FIG. 1. There is a statistically significant difference 40 min. after succinylcholine in the intact cat (p >.001), and between intact and adrenalectomized groups, 40 min. after succinylcholine (p >.001). Vertical lines represent S.E.

FIG. 2. Difference in blood glucose values after ether and pentobarbital sodium anesthesia was significant 60 min. in the rat (p >.01 <.02) and 20 min. in the cat (p >.05 <.02). Vertical lines represent S.E.

FIG. 3. Significant difference between groups was present 60 min. after reserpine (p >.05 <.02). Vertical lines represent S.E.

FIG. 4. All values are statistically significant (p <.05) except ADX C E at 45 min. (p >.2 <.1). Vertical lines represent S.E.

(5) it was of interest to evaluate the effect of ether and pentobarbital sodium on drug induced hypoglycemia.

Chlorpropamide was injected to cats and rats 3 hrs and 1 hr respectively before initiating anesthesia. Hyperglycemia resulted following ether, while pentobarbital sodium administered under same conditions did not interfere with lowered blood glucose (Fig. 2).

To evaluate further the importance of adrenal medullary hormones, cats were pretreated with reserpine prior to administration

TABLE II. Effect of Succinylcholine on Chlorpropamide Induced Hypoglycemia in Normal and Hepatectomized Rats.*

		Blood glucose, mg %				
	Drug	0′	30′	60′	90′	120′
Normal	Chlorpropamide, 100 mg/kg I.V.	98.6 ± 3.94†	77.1 ± 4.35	65.9 ± 5.86	75.8 ± 7.52	95.2 ± 8.63
Hepatectomized	*Idem*	77.8 ± 4.15	76.2 ± 3.23	62.2 ± 7.52	53.7 ± 3.74	51.8 ± 1.82

Succinylcholine administered after 60 min. * 6/group. † S.E.

of chlorpropamide. According to Holzbauer and Vogt(6) reserpine depletes the adrenals of epinephrine and norepinephrine. A lowering of blood glucose after chlorpropamide was obtained, however, when reserpine was injected after inducing hypoglycemia there was an immediate rise in blood glucose presumably caused by liberation of epinephrine (Fig. 3).

Epinephrine caused hyperglycemia in normal and in adrenalectomized rats(7) therefore, epinephrine and chlorpropamide were injected simultaneously to adrenalectomized and intact rats. There was complete suppression of hypoglycemic response in intact rat until 240 min when the antagonistic effect of epinephrine was no longer evident. Sensitivity of adrenalectomized rat to chlorpropamide as evidenced by mortality at 240 min was lessened by epinephrine (Fig. 4).

Discussion. Reversal of chlorpropamide induced hypoglycemia by succinylcholine in intact and its absence in the adrenalectomized animal, indicates influence of the adrenal gland. Our experiments point to the importance of epinephrine or norepinephrine as determining factors.

Dulin(8) observed that the role of adrenal cortex and medulla was important in modifying the hypoglycemic reaction of rats to tolbutamide. He also noted that epinephrine completely counteracted the hypoglycemic effects of tolbutamide.

It is noteworthy that hyperglycemic response was most pronounced in animals immobilized with succinylcholine, where the cortex was exposed for electroencephalographic recordings. Ether, which is said to stimulate the adrenal medulla *via* the pituitary adrenal axis(5) also elevated blood glucose in animals pretreated with chlorpropamide.

Adrenalectomized chlorpropamide treated rats responded with hyperglycemia to epinephrine injections, indicating that liver glycogen content was adequate for such response. To insure presence of sufficient glycogen in the liver, adrenalectomized cats were given cortisone. If glycogen is not available, as in the hepatectomized state, administration of succinylcholine will not result in hyperglycemia even though adrenals are present. This appears to indicate that succinylcholine causes a release of adrenal medullary substances, these in turn utilizing glycogen of liver resulting in hyperglycemic blood picture.

Ward and Dance(9) reported on the effect of succinylcholine on blood sugar in non-diabetic persons undergoing minor surgery. Succinylcholine caused but slight rise in concentration of blood sugar 60 min after administration of succinylcholine when used with pentothal sodium and nitrous oxide.

Summary. 1. In cats and rats, pentobarbital anesthetized and unanesthetized, a definite hypoglycemia was evoked with intravenously administered chlorpropamide. 2. Reversal of induced hypoglycemia was obtained wtih succinylcholine in intact rat and cat, but not in adrenalectomized cat. 3. Experiments performed with ether, reserpine and epinephrine, further supported the view that this antagonism was mediated through the adrenal gland.

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Uptake of Radiosulphate by Mucopolysaccharides of Aorta in Cholesterol Fed Cockerels. (25007)

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Several hypotheses have been proposed to explain the entrance of lipids into the arterial wall in the process of atherogenesis. Virchow suggested that the deposition of fat is secondary to the pathological alteration of connective tissue cells and ground substance of the vascular wall(1). This old idea seems to have gained importance in recent years. Faber(2) noted that alteration in the histochemical staining of arterial mucopolysaccharides is the earliest demonstrable feature of atherosclerotic lesions. He found that an increased output of carbohydrate-sulphuric acid esters plays a significant role in deposition of cholesterol in the vascular wall. Altschuler and Angevine(3) suggested that a process of ionic exchange between circulating cations and partially depolymerized chondroitin sulphuric acid may play a role in deposition and fixation of lipids. It is, therefore, apparent that depolymerization of mesodermal connective tissue ground substance is involved in the pathogenesis of atherosclerosis. This hypothesis is supported by more recent experimental work on cholesterol fed animals. Not only was hypercholesterolemia in rabbits associated with elevated serum hexosamine levels, but also increased metachromatic and P.A.S. positive materials were observed in and subjacent to atheromatous lesions(4). The above alterations of connective tissue ground substance also may be investigated by S^{35} uptake technic. It has been shown that radioactive sulphate may be incorporated into the aorta of animals and that the extent of uptake closely parallels intensity of staining for acid mucopolysaccharides. It was assumed that the principal substance incorporating S^{35} in the aorta was chondroitin sulphate(5,6). A recent report(7) on S^{35} uptake in the aorta of cholesterol-fed rabbits seems to confirm previous findings based on histochemical methods. Katz and Stamler(1), however, question the relevancy of rabbit cholesterol-induced aortic lesions for understanding the pathogenesis of human disease. They consider the chick as a more suitable experimental animal and give arguments in favor of this statement. Their advice, therefore, has been followed in selection of material for this work. The purpose of the present experiment was to study the effect of cholesterol feeding on uptake of S^{35} by the aortic wall mucopolysaccharides in cockerels.

Materials and methods. Cockerels of White Rock-Broiler strain used for this study were obtained from a commercial hatchery when 8 days old and immediately confined to thermostatic brooders. The birds were kept in groups of 8 to 10 per brooder and received routine chicken food with tap water as desired. When the birds were 2 weeks old cholesterol (2%) and cottonseed oil (5%) were added to the diet and this treatment continued during an 8 week period. Isotope was administered 24 hours before birds were killed. Radiosulphate (S^{35} in H_2SO_4, Atomic Energy, Canada) was injected subcutaneously in dosage of 1 mc/kg of bird's body weight. The dose was dissolved in 5 ml of distilled water together with 40 mg of sodium sulphate(8,9). The cockerels were anesthetized, killed by decapitation and the blood collected. Serum and livers were used for determination of lipids. From each bird, aorta and femur were carefully dissected for isotope study. Bone was studied to compare S^{35} uptake of the aorta with that of a tissue (bone) having specifically high sulphate-fixing capacity. Aortas were studied for S^{35} radioactive mucopolysaccharides following the method described previously(10,11). The bones were dissolved by the nitric-perchloric acid wet-digestion procedure described previously(12). The final precipitate of barium sulphate obtained after processing the tissues was transferred to planchettes and counting carried out using a thin mica window Tracerlab G-M tube. A Tracer-

TABLE I. Serum and Liver Lipids in Cholesterol Fed Cockerels, after 8 Weeks of Feeding.

	Control diet	Cholesterol diet
No. of birds	16	16
Serum total cholesterol, mg/100 ml	138 ± 6* (93–173)†	1143 ± 106 (507–1716)
Serum phospholipids, mg/100 ml	208 ± 5 (175–247)	380 ± 23 (250–527)
Serum, C/P‡	.67 ± .03 (.40–.82)	2.8 ± .12 (2.0–3.7)
Liver total cholesterol, mg/100 g	302 ± 15 (178–366)	10161 ± 428 (7681–13122)
Liver phospholipids, mg/100 g	2092 ± 73 (1440–2317)	3350 ± 54 (3040–3765)
Liver, C/P	.15 ± .1 (.08–.26)	3.0 ± .1 (2.3–3.5)

* Mean ± S.E.
† Range.
‡ Total $\frac{\text{cholesterol}}{\text{phospholipids}}$ ratio.

lab Superscaler S.C. 18A was used for reading. Results were expressed in counts per minute (c.p.m.). Total cholesterol was determined following the method of Abell *et al.* (13). For the study of lipid phosphate the samples were extracted with alcohol-ether and analysis carried out by the method of Zilversmit and Davis(14). Total cholesterol/phospholipid ratio (the C/P ratio) was obtained by calculation.

Results. Before the beginning of cholesterol diet body weights averaged 220 g and 232 g in control and cholesterol fed groups of birds respectively. At the end of experiment respective weights were 1680 g and 1570 g. Table I records data on determination of lipids in serum and livers of cockerels. Both total cholesterol and phospholipids are increased in cholesterol fed birds. Significant rise of C/P ratio, which is considered as a decisive factor in atherogenesis of cockerels(15) also may be noted. Table II presents the results of measuring of S^{35} uptake by the fraction of aortic tissue considered to contain sulphated mucopolysaccharides. Total radioactivity of femora of the same birds is also recorded. Significant difference between the 2 groups of cockerels is apparent as far as aortic S^{35} uptake is concerned. There is no marked difference in S^{35} uptake by the bones of these birds.

TABLE II. Uptake of S^{35} by Bone and Radioactivity of S^{35} Sulphated Mucopolysaccharides of Aortas (S.M.) in Cockerels.

	Control diet	Cholesterol diet
No. of birds	16	16
S^{35} in bone, c.p.m./100 mg dry wt	942 ± 43* (720–1305)†	987 ± 47 (713–1276)
S^{35} in S.M. fraction of aorta, c.p.m./100 mg dry wt	762 ± 45 (537–999)	1612 ± 106 (1162–2362)

* Mean ± S.E.
† Range.

Discussion. Increased S^{35} uptake by the sulphated mucopolysaccharides of the aortic wall in cholesterol fed cockerels was found in this experiment. This finding is in agreement with previous work on alteration of vascular wall ground substance associated with clinical and experimental atherosis. The fact that cholesterol feeding does not influence S^{35} uptake by bones is significant. It means that the changes in lipid metabolism produced under described experimental conditions do not necessarily result in increase of the S^{35} uptake by all tissues with sulphate-binding capacity.

Summary. Cockerels were fed during an 8 week period with cholesterol (2%) and cottonseed oil (5%) enriched diet. Parallel to increase of serum and liver lipids and C/P ratio, there was a significant rise of S^{35} uptake by the sulphated mucopolysaccharides of the aorta.

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Comparative Effects of Bile Acids on Intestinal Absorption of Cholesterol.* (25008)

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Schoenheimer and co-workers reported the first critical experiments on facilitatory effect of bile salts on cholesterol absorption in various animals(1-3). Using serum or liver cholesterol levels as indicators of quantitative differences between bile acids, these workers found that glycocholate had the greatest effect in enhancing cholesterol absorption, while cholic acid and desoxycholic acid were less effective(2,3). Member *et al.*(4) reported that cholate or glycocholate fed with cholesterol to rabbits increased blood and aorta cholesterol levels, while dehydrocholate, hyodesoxycholate, and desoxycholate were ineffective. In chronic feeding experiments with rats, Swell and co-workers(5) found that blood cholesterol levels were elevated by inclusion of cholate or taurocholate in high fat-high cholesterol diets; desoxycholate was less effective, while dehydrocholate was without noticeable effect. Correlation of these data with *in vitro* studies on comparative effects of different bile salts on cholesterol esterase activity(6) led to the suggestion that the observed effects of dietary bile salts were due to their requirement as cofactors for cholesterol esterase activity. Another explanation is that due to hydrotropic properties of these substances(7), dispersion of cholesterol and its tranfer into the mucosa is increased. Recently, Swell and coworkers(8) demonstrated that bile salts are involved in passage of cholesterol from the intestinal lumen into the mucosa. Further, these authors suggested that a specific type of bile salt was involved, since dehydrocholate was ineffective in promoting transfer of cholesterol into the intestinal mucosa, while taurocholate increased both mucosal and lymph cholesterol levels(8). Other workers have suggested that accumulation of cholesterol in blood and liver when bile salts are fed is related to depressed rate of cholesterol catabolism or excretion(9,10). The purpose of the present investigation was to determine directly, using changes in lymph cholesterol levels, the comparative effects of various bile salts on intestinal absorption of cholesterol in the rat.

Methods and materials. Male rats of Carworth Farms strain with thoracic duct lymph fistula were prepared and handled as described previously(11). Following a fasting period of 18-24 hours, the animals were given 3 ml of aqueous test emulsion intragastrically (11). Control and test emulsions contained 50 mg of albumin, 50 mg of cholesterol and 288 mg of oleic acid/3 ml of 0.9% saline. In addition, the experimental emulsions contained 4 molar equivalents of one of 7 different bile acids/mole of cholesterol fed. Commercial samples of lithocholic acid, deoxycholic acid, dehydrocholic acid, sodium taurocholate and sodium glycocholate (Nutritional Biochemicals) were employed. In addition, highly purified samples of cholanic acid,† cholic acid‡ and synthetic

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† Mann Research Lab., M.P. 163.5°C; sp. rot., + 21°.

‡ We are indebted to Dr. M. Korzenovsky, Eli Lilly and Co., for sample of purified cholic acid.

TABLE I. Effect of Various Bile Acids on Absorption of Cholesterol in 9 Groups of Lymph-Fistula Rats.

Additions	No. of rats	Avg lymph vol/24 hr, ml	Lymph cholesterol/24 hr		
			Total, mg	Free, mg	Ester, %
None*	4	57 ± 9	21 ± 2	5 ± .2	76 ± 2
Cholanic acid	3	53 ± 5	20 ± 2	5 ± .3	74 ± 1
Lithocholic acid (3-hydroxycholanic acid)	4	72 ± 6	20 ± 2	6 ± .8	72 ± 3
Deoxycholic acid (3,12-dihydroxycholanic acid)	4	51 ± 5	24 ± 2	6 ± .6	77 ± 1
Cholic acid (3,7,12-trihydroxycholanic acid)	5	118 ± 29	40 ± 3†	7 ± .6†	82 ± 1†
Dehydrocholic acid (3,7,12-triketocholanic acid)	5	69 ± 12	21 ± 1	6 ± .4	74 ± 0
Glycocholate	4	132 ± 22†	32 ± 3†	7 ± .2†	77 ± 2
Taurocholate	5	94 ± 24	33 ± 2†	7 ± .2†	80 ± 2†
Synthetic taurocholate	5	99 ± 23	33 ± 1†	6 ± .5	82 ± 1†

* Basic emulsion for all groups contained 50 mg cholesterol, 50 mg albumin, and 288 mg oleic acid/3 ml of .9% saline/rat(15).

† $p < .05$ for difference between experimental and control group.

sodium taurocholate(12) were also used. Lymph was collected for 24 hours after administration of test emulsions and frozen until analyzed for total and free cholesterol(13). Values for esterified cholesterol were obtained by difference. There were 3-5 animals/group, and apparent differences between control and experimental groups were analyzed for significance by the t-method of Fisher(14). Probability levels of $p<0.05$ were considered significant.

Results. Previous investigations of effects of different bile acids on cholesterol absorption have been long-term in nature and have employed variations in blood and liver cholesterol levels as indicators of cholesterol absorption from the intestine. In these approaches there is the possibility that increases in tissue sterol levels are due to effects on cholesterol metabolism in liver or other tissues (9,10) and do not specifically measure intestinal absorption. The present approach is more suitable for studying the effect of bile acids directly on cholesterol absorption since thoracic duct lymph is the primary transport route for absorbed cholesterol(16,17); also a single gastric feeding has been employed eliminating any long-term effects of bile acids.

The results (Table I) show that cholanic acid, lithocholic acid (3-hydroxycholanic acid) and deoxycholic acid (3,12-dihydroxycholanic acid) have no direct effect on absorption of cholesterol from the intestinal tract. These results are in general similar to those of earlier studies based on measurements of blood and liver cholesterol levels. However, those technics have generally shown a significant effect by deoxycholic acid; this was not found in our study. Thus, long-term feeding of deoxycholic acid in diets containing cholesterol produces changes in tissue cholesterol levels which are not due directly to increased absorption of dietary cholesterol. Administration of cholic acid (3,7,12-trihydroxycholanic acid) with cholesterol and oleic acid gave an increase in total lymph cholesterol of 19 mg/24 hours, which was equivalent to 38% of that fed. This facilitatory effect of cholic acid on cholesterol absorption was greater than with other bile acids tested. Thus, it appears that presence of 3 hydroxyl groups on the bile acid is necessary for the activity in promoting cholesterol absorption, since removal of even one, as in deoxycholic acid, results in a loss of this effect. Also, dehydrocholic acid, containing 3 keto groups, had no effect in promoting cholesterol absorption.

Comparison of effects of taurocholate or glycocholate on cholesterol absorption with that of cholic acid shows that esterifying the free carboxyl group of the bile acid depresses its effect on cholesterol absorption. The type of amino acid conjugated to cholate is not critical since both taurocholate and glycocholate had the same quantitative effect on absorption of cholesterol.

Differences in lymph free cholesterol between control and experimental groups were insignificant except for those fed cholic acid, glycocholate, and commercial taurocholate. In these groups where cholesterol absorption occurred, values for free cholesterol were greater than in the control group; this relationship has been discussed(11). The data clearly demonstrate that certain bile salts have a direct effect on intestinal absorption of cholesterol in addition to the reported effect of these substances on hepatic metabolism of cholesterol(9,10).

Summary. 1. Effect of various bile acids on intestinal absorption of dietary cholesterol has been investigated in lymph-fistula rats. 2. Cholanic, lithocholic, and deoxycholic acids, with none, one and 2 hydroxyl radicals, respectively, produced no increase in lymph cholesterol over the control group. 3. Conjugated bile salts, glycocholate and taurocholate gave comparable significant elevations in total lymph cholesterol/24 hours. 4. Cholic acid with 3 free hydroxyl groups and an unconjugated carboxyl radical was the most effective bile acid in promoting cholesterol absorption from the intestine.

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Fluorescent-Antibody Technic for Serodiagnosis of *Trypanosoma cruzi* Infection.* (25009)

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Since Coons and his colleagues first reported the immuno-specific staining of tissue with fluorescein-conjugated globulin(1), numerous applications of the technic have been found. It was shown that fluorescein-labeled antibody not only provided a means of demonstrating localized antigen in tissues, but could be used to detect specific antibody developed during the course of infection with certain micro-organisms, *e.g.*, *Toxoplasma gondii*(2) and *Treponema pallidum*(3). The present report describes a fluorescent-antibody test developed for serodiagnosis of American trypanosomiasis (Chagas' disease), and gives an improved method for purifying antibody prior to conjugation with fluorescein. The procedure was evaluated in tests with homologous and heterologous sera and the results compared with those obtained in complement fixation tests.

Materials and methods. The indirect technic, using a labeled antihuman antibody, was employed. *Fluorescein conjugate*: Labeled anti-antibody was prepared by conjugating the euglobulin fraction(4) of anti-human gamma globulin horse serum with fluorescein isothiocyanate[†] according to the method of Marshall *et al.*(5). The euglobulin fraction was precipitated from whole serum by slowly adding, with constant stirring, 9 volumes of 0.0027 N HCl to 1 volume of chilled antiserum, and incubating the mixture at 1°C for 30 minutes to assure maximum precipitation. The euglobulin precipitate was sedimented by cold temperature (1°C) centrifugation for 10 minutes at 1000 g, and reconstituted to one-fourth of the original serum volume with 0.02 M phosphate-buffered salt solution, pH 6.8; trace amounts of insoluble residue were removed by centrifuging for 10 minutes at 1000 g. For conjugation, the euglobulin solution was diluted with sodium chloride solution (0.15 M) and carbonate-bicarbonate buffer (0.5 M, pH 9) so that the final solution contained 10 mg protein/ml and 10% buffer. The required quantity of fluorescein isothiocyanate (0.05 mg dye/mg protein) then was added to the alkaline euglobulin solution and the mixture stirred with magnetic stirrer for 18 hours at 3°C. Uncoupled fluorescein was removed by dialysis in the cold against successive changes of 0.01 M phosphate-buffered salt solution, pH 7.0 (PBS). Insoluble components of the dialysant were sedimented by centrifugation and the clear, labeled antibody solution stored at 3° or –20°C. Prior to use, each lot of labeled antibody was titrated with homologous and normal sera. The highest dilution of conjugate giving brilliant fluorescence in tests with *T. cruzi* antiserum and essentially no fluorescence with normal serum was used in the diagnostic tests; in most instances, a 1:10 or 1:20 dilution satisfied these conditions. *Sera*: Since the indirect technic using labeled anti-human antibody was employed, test sera necessarily were of human origin. Sera representing *T. cruzi* infection were from well documented cases, the diagnoses having been established either by demonstrating the organism in stained blood films, or by xenodiagnosis. The syphilitic sera were from patients with *T. pallidum* infection, whose diagnoses were confirmed by the *T. pallidum* immobilization (TPI) test. Normal sera were obtained from healthy donors undergoing physical examination as candidates for appointment to U.S. Military Academy; all were negative in the TPI test. The sera were stored at –20°C. Immediately before testing, *ca* 0.2 ml of thawed serum was heated for 30 minutes in water bath at 56°C. *Test procedure*: All reactions were carried out in test tubes to prevent drying of organisms. Cultures of *T. cruzi* (Brazil strain)[‡] grown on a diphasic medium(6) for 8-12 days at 23°C served as source of antigen. The organisms were freed from debris by differential centrifugation and washed once with formalinized salt solution (0.15 M sodium chloride containing 0.1% formalin) and once with PBS. A PBS suspension containing 10^7 washed trypanosomes/ml was the standardized antigen. For each test, 1 ml of antigen suspension was transferred to 13 x 100 mm test tube and centrifuged 10 minutes at 1000 g. Following decantation of supernate, 0.1 ml of test serum was added to the sedimented trypanosomes, mixed, and incubated for 18 hours at 3°C. The organisms then were washed twice with PBS, sedimented, and treated with 0.1 ml of diluted fluorescein-labeled anti-human antibody. After incubation for 30 minutes at 3°C, the trypanosomes again were washed and finally suspended in 0.1 ml of PBS. A small drop (*ca* 0.02 ml) of test mixture was combined with equal volume of 90% buffered glycerine on a micro-slide, surmounted with a glass coverslip, and examined under fluorescent microscope. Degree of fluorescence was estimated according to criteria similar to those of Goldman(2), and results classified as: reaction (4+, 3+, 2+), weak reaction (1+), or no reaction (±, –). *Fluorescence microscope*: A standard monocular microscope fitted with first-surface mirror, reflecting dark field condenser, and a Corning #3496 yellow filter in the ocular was employed. The ultra-

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[‡] Obtained through courtesy of Eleanor J. Tobie, Lab. of Tropical Dis., N.I.H., Bethesda, Md.

TABLE I. Relative Sensitivity and Specificity of Fluorescent-Antibody and Complement Fixation Tests for *T. cruzi* Infection.

	Persons exhibiting the given serologic result* in							
	Fluorescent-antibody tests				Complement fixation tests†			
Diagnostic status	No. tested	Reaction	Weak reaction	No reaction	No. tested	Reaction	Weak reaction	No reaction
Chagas' disease	40	39	1		40	28	9	3
Syphilis	20		2	18	49			49
Healthy	70		3	67	100			100

* Classified as reaction (4+, 3+, 2+), weak reaction (1+), or no reaction (±, —).

† Tests with purified protein antigen fraction derived from *T. cruzi*.

violet light was supplied by AO-Spencer fluorescence illuminator #633 using Osram HBO-200 mercury arc lamp; a Corning #5850 blue filter was used for transmitting light in the near ultraviolet wavelength.

Results. Sensitivity and specificity of the fluorescent-antibody reaction was evaluated in tests with homologous, syphilitic, and normal sera, and the results compared with those obtained in complement fixation tests using a purified protein antigen derived from *T. cruzi* (7). The findings are summarized in Table I. Tests with 40 sera obtained from well documented cases of *T. cruzi* infection illustrate the relative sensitivity of 2 procedures. All sera reacted serologically in the fluorescent-antibody test, whereas only 37 of 40 specimens gave reactions in the complement fixation test. Results obtained with syphilitic and normal sera, on the other hand, provided an index of relative specificity of test procedures. Two of 20 syphilitic and 3 of 70 normal sera gave weak reactions in the fluorescent-antibody tests. In contrast, the complement fixation reactions were uniformly negative.

Discussion. The present report is comprised of studies on 2 discrete but related problems. One deals with development of a serologically specific fluorescent-antibody procedure, the other with an improved method for purifying antibody to be labeled. It was found early in the present studies that dried smears of *T. cruzi* were unsatisfactory for fluorescent-antibody tests, although other investigators successfully have used fixed smears of micro-organisms for a variety of fluorescent-antibody procedures. With *T. cruzi*, dried unfixed smears as well as those treated with various concentrations of methanol, ethanol, formalin, or with heat regularly gave nonspecific reactions with normal sera. The untoward reactions, moreover, were not affected by preliminary treatment of smears with crystalline bovine serum albumin solution. Efforts to improve the specificity of staining by using the "combined" or "sequence" inhibition (blocking) technics employed by Goldman in studies on toxoplasmosis(2) likewise were unsuccessful. Continued investigations revealed that non-specific staining was associated with desiccation of the trypanosomes. Solution of the problem therefore was found in tests conducted in test tubes wherein drying of the organisms was prevented. With the indirect test procedure that evolved from these studies, *T. cruzi* antisera readily could be differentiated from syphilitic or normal sera. In tests with homologous antisera, the organisms fluoresced brilliantly and exhibited the typical yellow-green color of fluorescein. Conversely, the trypanosomes were only faintly visible in tests with heterologous sera, and the color was a nondescript yellow-orange rather than the yellow-green characteristic of the specific reaction.

Serologic evaluation with homologous, syphilitic, and normal sera showed the fluorescent-antibody test to be superior in sensitivity, but slightly less specific than the complement fixation test using a purified *T. cruzi* antigen (Table I). On the other hand, specificity of the fluorescent-antibody procedure was greatly superior to that exhibited in complement fixation tests using the commonly employed unpurified *T. cruzi* antigens; *e.g.*, in earlier studies(8) a buffered-saline extract of *T. cruzi* reacted with 7 of 75 normal sera and 9 of 21 specimens from syphilitic donors. The findings suggest that the fluorescent-antibody test may provide a relatively simple, reliable procedure for diagnosis of *T. cruzi* infection and be of particular value to laboratories not having access to purified complement fixing antigen. Furthermore, the fluorescent-antibody test, in view of its high degree of sensitivity, appears especially well suited for use as a screening procedure and could reduce the number of sera requiring study with the slightly more specific but intricate complement fixation test.

The need for further evaluation of the fluorescent-antibody procedure is evident since heterologous sera representing parasitic diseases other than American trypanosomiasis were not available during the present experiments. Moreover, any further studies should consider the possible improvement of specificity by arbitrarily reducing the sensitivity level of the test procedure. Investigations to determine whether the same antibodies are responsible for the complement fixation and fluorescent-antibody reactions likewise seems a worthwhile subject for future study.

With regard to preparation of fluorescein-labeled antibody, many investigators contend that the quality of conjugates can be improved by removing non-antibody serum components before labeling. Usually this is accomplished by repeated precipitation of the antiserum with half-saturated ammonium sulfate. The resultant antibody-containing globulin fraction then is tagged with fluorescein. Certain inherent problems, however, are encountered with this procedure. It is not only time-consuming, but requires extensive dialysis for complete removal of residual ammonium sulfate. In addition, the globulin fraction undergoes considerable dilution during dialysis, and frequently must be concentrated before it can be labeled. These difficulties were obviated in the present studies by isoelectric precipitation of the serum euglobulin fraction with dilute HCl(4). Specific staining capacity of euglobulin-fluorescein conjugate repeatedly was observed to be significantly greater than that exhibited by labeled ammonium sulfate-precipitated globulin or whole serum. The superiority of the euglobulin fraction was further indicated by the relatively small amount of precipitate that developed during conjugation, suggesting little if any gross alteration of antibody components.

Summary. A fluorescent-antibody technic for serodiagnosis of American trypanosomiasis is presented. While nonspecific staining regularly occurs in tests using dried smears of trypanosomes, specificity is excellent when reactions are conducted in test tubes and drying of organisms is prevented. Serologic findings indicate the fluorescent-antibody procedure, used either independently or in conjunction with the complement fixation reaction, can play an important role in serodiagnosis of *T. cruzi* infection. An improved method for purifying antibody prior to conjugation with fluorescein also is given. Advantages of purification by isoelectric precipitation with dilute HCl over ammonium sulfate as a precipitant are noted.

The authors are indebted to Dr. J. F. Kent for suggesting the use of serum euglobulin fraction in preparing fluorescein-labeled antibody.

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Phenolic Steroids in Human Subjects III. Estrogens in Plasma of Pregnant Women.*† (25010)

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Data on concentration of the various estrogens in human plasma during pregnancy are meager. Furthermore, much of the work was carried out with methods lacking sensitivity and specificity for the low concentrations of these steroids in plasma. Aitken and Preedy (1) determined levels of circulating estrogens during the last 4 weeks of pregnancy and found the following values, expressed as μg/100 ml of plasma: estrone, 2.6-10.3; estradiol-17β, 1.2-2.9; estriol, 4.3-17.5. In discussion following the paper(1) Loraine quoted unpublished data of Roy and Brown who found approximately one-half as much estrogen in plasma as Aitken and Preedy. Diczfalusy in discussing the same paper(1) reported concentrations obtained in "so-called retroplacental blood, *i.e.*, on vaginal blood following delivery." His values, expressed as μg/100 g of blood, were as follows: unconjugated-estrone, 1-3, estradiol-17β, 2-6, estriol, 3-13; conjugated-estrone, 1-4, estradiol-17β, 0.1-0.3, estriol, 4-20. In this paper data on estrogen levels in human plasma during the second and third trimesters of pregnancy are presented. Identity of various estrogens has been established by isotopic dilution and by paper chromatography.

Methods and materials. Approximately 30 ml of blood were drawn from a number of pregnant women and mixed with heparin. Plasmas were separated from erythrocytes, pooled and stored frozen (–10°C) until 1-2 liters were accumulated. After thawing, proteins were precipitated by adding ethanol to final concentration of 80%. After standing at 5°C overnight, precipitate settled leaving a clear layer. The latter was drawn off and the residue centrifuged. After washing precipitate with twice its volume of absolute ethanol, combined supernatants were filtered and ethanol was removed *in vacuo* at a temperature less than 40°C. Radioactive estrogens (0.02 μC, 1-9 μg of 16-C^{14}-labelled estrone, estradiol and estriol) were added to 2 of the deproteinized plasmas at this point. (In the other case they were added just before countercurrent distribution.) Upon standing overnight at 5°C, fat rose to the top and solidified. It was lifted out and discarded. Deproteinized plasma was extracted twice with equal volumes of hexane and the hexane discarded. Residual hexane in the aqueous phase was blown off with air. The deproteinized plasma, adjusted to pH 5 with acetate buffer, was incubated with β-glucuronidase (1000 units of Ketodase/ml) for 72 hours at 37°C. After pH had been lowered to 1 with 60% sulfuric acid, continuous extraction with ether was performed for 48 hours. Concentrated hydrochloric acid (0.15 volume) was added to the boiling aqueous solution and the mixture refluxed for 45 minutes. The cooled hydrolyzate was extracted 3 times with ¼ volume of ether. The ether was evaporated and the residue, combined with that from continuous ether extraction, was dissolved in 100 ml of ethyl acetate, washed twice with ⅕ volume of saturated sodium bicarbonate solution and once with 1/10 volume of water. The ethyl acetate was removed *in vacuo*. The residue was separated by 99 transfer countercurrent distribution in the system 70% methanol/60% carbon tetrachloride-40% chloroform. Analysis of both fluorescence(unpublished) and radioactivity was performed(2). The contents of tubes under each peak were pooled. Infrared spectrophotometry was attempted, but the fractions were too impure. Consequently, each pool was chromatographed on paper in the systems benzene/55% methanol, or 33% benzene-67% Skellysolve C/80% methanol. Each chromatogram was scanned in an Actigraph for localization of radioactive

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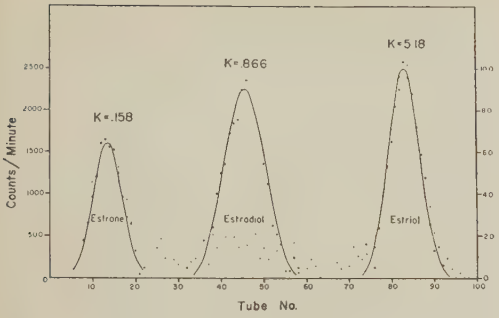

FIG. 1. Countercurrent distribution of an extract of 1.8 liters of plasma from women 28-35 wk pregnant. System: 70% methanol/60% carbon tetrachloride-40% chloroform. × radioactive assay, ● fluorimetric assay. Solid curves are theoretical curves drawn to the partition coefficients (K) given above each curve.

estrogens. One-half of the paper strip was developed colorimetrically with Folin-Ciocalteu reagent(3). Appropriate areas from the other half of strip were eluted, dried, and the residues acetylated with acetic anhydride and pyridine at room temperature overnight. Estrone acetate was rechromatographed and the Zimmermann reaction used to localize the steroid. Estradiol diacetate and estriol triacetate was partially saponified in 0.5 N methanolic potassium hydroxide at room temperature for 30 seconds. The solution was neutralized with acetic acid, diluted with water and extracted with ether. This procedure removes the phenolic acetate group, making the compounds again reactive to the Folin-Ciocalteu reagent. The partially acetylated compounds were chromatographed.

Results. A countercurrent distribution of plasma extract is shown in Fig. 1. In general, there was excellent correlation of radioactivity and fluorescence (except for estradiol in the instance shown), the latter expressed in μg of each estrogen. Analysis of countercurrent distribution data by the method of Baggett and Engel(4) also indicated identity of endogenous hormone and added radioactive steroid. Paper chromatography of the alcohol and its acetate indicated in each case the identity of the radioactive and endogenous steroid. Thus, although we failed to obtain adequate infrared spectra, the identification of the compounds is adequately established.

Two women in each group were taking diethylstilbestrol, but this compound does not fluoresce. In addition, these women contributed less than 2% of the plasma analyzed. None of the women was taking ethinylestradiol, which does fluoresce and which would be difficult to distinguish from estradiol by countercurrent distribution. There were also other fluorogens present. There was a very definite shoulder at tubes 20-25 in each of the specimens studied. In the case of the second trimester plasma extract, there was also a pronounced peak at tube 93. Between the estradiol and estriol areas a varying amount of fluorescence was present in all the extracts examined. It is not known whether these fluorogens are steroidal.

Results of 3 experiments are tabulated in Table I. Concentrations of each of the 3 estrogens were calculated in 2 ways: on the basis of total area of each fluorescent curve and by dilution of added isotopic estrogen. There was excellent agreement of the 2 methods. The amount of estrone and estradiol obtained from plasma collected during the last 4 weeks of pregnancy agrees with the values reported by Aitken and Preedy(1), but our value for estradiol is higher. Our values for concentration of estrone and estradiol during this period are consistent with those of Diczfalusy for whole blood(1), but our estriol value is lower.

It is of special interest to note the relative constancy of estrone and estriol concentrations during the last 2 trimesters of pregnancy, especially in view of the 10- to 100-fold increase in urinary estrogens during this period (5). Estradiol concentration, on the other hand, was quite variable. There was a small amount of estradiol present in the extract from the second trimester plasma, none during the first part of the third trimester (Fig. 1), and a large amount during the last 4 weeks of

TABLE I. Concentration of Estrogens in Human Pregnancy Plasma.

Period of pregnancy, wk	Vol of plasma processed, ml	μg/100 ml of plasma		
		Estrone	Estradiol-17β	Estriol
14-27	2200	5.1	<1.4	4.3
28-35	1800	3.1	< .1	5.1
36-40	1100	9.3	8.2	6.1

pregnancy (Table I). We have no explanation for this.

Summary. By means of countercurrent distribution and paper chromatography, estrone, estradiol and estriol were separated from extracts of one to 2 liters of human pregnancy plasma, quantitated and identified by fluorimetric and isotopic technics. Concentration of estrone and estriol remained more nearly constant (3-9 μg/100 ml of plasma) through second and third trimesters, but the concentration of estradiol varied from 0-8 μg/100 ml.

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Enhancement of Lethal Action of Endotoxin in Mice by Triiodothyronine. (25011)

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Mice survive lethal doses of endotoxin when pre-treated with cortisone(1). Consideration of interrelationship between thyroid hormone and resistance of mice to endotoxin resulted from observations in dogs in that cortisol production and metabolism were significantly increased by analogs of thyroxin, such as triiodothyronine and triiodothyroacetic acid. In our studies for publication it was found that the magnitude of the rise in cortisol production simulated secretory response of the adrenal to exogenous corticotropin. Others(2) previously reported that following injection of 1-thyroxine into guinea pigs urinary output of 17 hydroxycorticosteroid was in direct proportion to dose of thyroxine. Because triiodothyronine increased output of cortisol in the intact animal, it was anticipated that its administration to mice would protect them against lethal action of endotoxin, but on the contrary, triiodothyronine enhanced this lethal action.

Methods. Purified endotoxins were prepared from cultures of *Escherichia coli, Salmonella typhosus* and *Brucella abortus*, as described elsewhere(1). Male ABC mice weighing approximatley 20 g were given endotoxin intraperitoneally in dose of 0.2 to 0.4 mg/20 g body weight. The dose of endotoxin selected for each experiment was that amount which did not produce death in normal mice within 48 hours. A solution of 3:5:3′-1-triiodothyronine was prepared to yield a concentration of 40 μg/0.1 ml.* Groups of 10 mice each were used in a series of experiments. In the first experiment, 20 mice were given 40 μg of triiodothyronine intraperitoneally, and 18 hours later this dose was repeated, a total of 80 μg within 24 hours. Preliminary metabolic studies revealed that within 6 to 12 hours after final injection metabolism was accelerated, reaching a peak within 24 hours. Then, 10 mice served as controls, and the 10 remaining animals received intraperitoneally 0.3 mg/20 g of *E. coli* endotoxin. An additional group of 10 mice received only endotoxin. In a second series of experiments the same procedure was carried out except that 0.4 mg/20 g of endotoxin prepared from *Br. abortus* was used. Similarly, in the third series, 0.2 mg/20 g of endotoxin made from *S. typhosus* #360 was injected.

Results. Results of 3 experiments using 3 different preparations of endotoxin are presented in Table I. Triiodothyronine consistently enhanced the lethal action of endotoxin. Deaths were recorded as having taken place within 48 hours after mice had received endo-

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* Supplied through courtesy of Smith, Kline and French Labs., Philadelphia, Pa.

TABLE I. Enhancing Effect of Triiodothyronine on Lethal Action of Endotoxin in Mice in 3 Experiments.

Source of endotoxin	Deaths within 48 hr* Endotoxin	 Triiodothyronine and endotoxin
E. coli	1/10	9/10
Br. abortus	0/10	6/10
S. typhosus	1/10	7/9
Total	2/30	22/29

* Numerator signifies No. dead.

toxin. Mice surviving usually do not succumb to lethal effect after this period. The majority of animals in our experiments died within 24 hours after having endotoxin injected into them. None of the control mice receiving only triiodothyronine died. There were no unusual gross lesions of organs in animals dying within 48 hours. The outstanding feature summarized in Table I is that only 2 out of 30 mice receiving endotoxin alone died, whereas 22 of 29 mice receiving both triiodothyronine and endotoxin died.

Comment. Cortisone has consistently protected mice against the lethal effect of endotoxin. Since it had been established in the intact dog that triiodothyronine administration resulted in an increase in cortisol production, it was anticipated that enhancement of metabolism in the mouse by triiodothyronine would afford protection against endotoxin. On the contrary, the lethal action of endotoxin was accelerated shortly after administration of triiodothyronine. The mechanism whereby triiodothyronine enhances lethal action of endotoxin is not understood. It could be postulated that the induced state of hyperthyroidism greatly accentuated inactivation of steroid so that adrenal steroid synthesis was exceeded, and adrenal insufficiency occurred even in presence of maximal adrenal secretory activity. In dogs pretreated with analogs of thyroxine, rate of disappearance of cortisol from plasma is greatly accelerated. Furthermore, in humans with thyrotoxicosis rate of removal of cortisol from plasma is markedly accelerated(3). Removal of cortisol from plasma is a reflection of rate of catabolism of the hormone by the liver. *In vitro* reduction of ring A (inactivation) of cortisone by Δ^4-steroid hydrogenases of rat liver is enhanced by triiodothyronine(4).

A second and more plausible explanation relates to the basic effect of endotoxin on vascular activity. Immediately after administration of endotoxin a severe degree of vasoconstriction takes place. This in turn results in a state of anoxia involving certain organs and tissues. Since we have observed that triiodothyronine markedly increases oxygen consumption of mice, it is most likely that the increased demands for oxygen induced by both endotoxin and by triiodothyronine account for the accelerated death rate.

Summary. Triiodothyronine enhances the lethal action of endotoxin in mice. It is postulated that this enhancement is associated with anoxia, caused by vasoconstriction and subsequent hypotension that is induced by the endotoxin; and the increased demand for oxygen that is produced by triiodothyronine.

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Some Effects of Enzymatic Digestions of Fixed Monkey Kidney Tissue Cultures on their Staining Patterns.* (25012)

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Nearly a half century ago Van Herwerden (1) used a spleen extract containing native ribonuclease to diminish basophilia of sea urchin oöcyte. Many others (see, *e.g.*, 2, 3, 4) have since used ribonuclease and other enzymes to digest cells before staining and microscopic examination, with results sometimes very useful for determining intracellular distribution of cellular materials. The purpose of this paper is to report results obtained by applying such enzymatic digestions to a primate cell grown in tissue culture. Specifically, the effects of digestion of fixed monkey kidney cells with chymotrypsin, ribonuclease, desoxyribonuclease, and some other enzymes on subsequent staining pattern of these tissue culture cells will be given.

Materials and methods. Sources of enzymes and stains. Chymotrypsin (salt free from ethyl alcohol), desoxyribonuclease (1x crystallized), lipase (wheat germ), pancreatin (3x U.S.P. potency), papain, ribonuclease (crystalline), and trypsin (2x crystallized, containing 50% $MgSO_4$) were obtained from Nutritional Biochemicals Corp., Cleveland, O., Trypsin (1:250) from Difco Laboratories, Detroit, Mich. Pyronin Y and thionin were obtained from National Aniline Division, Allied Chemical and Dye Corp., N. Y., Basic fuchsin from Coleman and Bell Co., Norwood, O. *Purification of pyronin Y and thionin.* For some cultures pyronin Y was used without purification; for other cultures purified preparations were used. Paper chromatography of commercial pyronin Y in solvent composed of 3 volumes *i*-propanol and 1 volume N aqueous formic acid revealed that this stain contained at least 3 colored compounds in addition to pyronin itself, the major rose red constituent at Rf 0.59. Pyronin was partially separated from the other 3 colored compounds (purple one at Rf 0.77, rose at Rf 0.26, and mauve at Rf 0.00) by extracting an aqueous solution of commercial pyronin Y 3 times serially, each time with 0.4 volume *n-amyl* alcohol. These 3 alcoholic extracts were combined and extracted once with 0.11 volume demineralized water. This water extract was shown by paper chromatography to contain the major rose red component largely but not entirely free of the other 3 colored compounds and is referred to herein as purified pyronin. Paper chromatography of commercial thionin in 3 volumes *i*-propanol and 1 volume N aqueous ammonia revealed, in addition to blue spot of thionin itself at Rf 0.40, a less intensely colored mauve spot at Rf 0.69. The blue compound was then separated from the mauve compound by adding 0.05 volume N aqueous ammonia to aqueous solution of the impure material. This ammoniacal solution was then extracted 4 times serially, each time with equal volume of ethyl acetate. Thionin then crystallized upon partial evaporation of final aqueous phase. This crystalline product showed none of the mauve component by paper chromatography and is referred to herein as purified thionin. For some cultures this purified thionin was used whereas for others the impure commercial product was used. *Preparation of fixed monkey kidney tissue cultures*: Kidney cells from rhesus (*Macaca mulatta*) or cynomolgus (*Macaca irus*) monkeys were dispersed with trypsin using the technic of Youngner(5). These cells were grown on cover slip fragments (*ca* 1 cm x 1 cm) placed in 60-mm diameter Petri plates. Growth medium was lactalbumin medium of Melnick(6) and the Petri plates were incubated at 35-37°C in incubator continuously flushed with 3% CO_2 and 97% air. Cover slip fragments with adherent cultures of kidney cells were removed after cells had grown for 3-6 days, at which time usually 30-90% of top surface of fragment was covered with

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TABLE I. Effects of Digestion of Fixed Monkey Kidney Cells with Chymotrypsin, Ribonuclease, and Desoxyribonuclease on Their Subsequent Staining with Pyronin, Thionin, and Feulgen Method.

		Effect of enzymatic digestion of			
		Formalin-fixed cells			Carnoy's-fixed cells
		On staining with			
Enzyme	Portion of cell	Pyronin[*]	Thionin[*]	Feulgen method	
Chymotrypsin	Nucleus	0[†]	0[†]	0	Cells removed[‡]
	Cytoplasm	DD	DD	0	
Ribonuclease	Nucleus	D[§]	0[†]	0	0
	Cytoplasm	DDD	DDD	0	0
Desoxyribonuclease	Nucleus	DD[‖]	DDD[‖]	DD[‖]	
	Cytoplasm	0	0	0	Not done

0, no effect; D, slight decrease in intensity of staining; DD, moderate decrease; DDD, large decrease.

[*] Purified stain in M/10 sodium acetate buffer, pH 4.2.

[†] Whether or not nucleolar staining was decreased is not known since these results are from cultures treated with hypotonic buffered saline and nucleoli of cultures so treated are usually not readily identifiable.

[‡] Chymotrypsin removed Carnoy's-fixed cells from cover slip.

[§] This is actually a moderate decrease in nucleolar staining without noticeable decrease in staining of rest of nucleus.

[‖] Staining of mitotic chromosomes was similarly diminished.

cells. These freshly harvested cultures were thoroughly washed in phosphate-buffered saline (PBS)(7) at pH 7.2-7.4. Those cultures whose chromosomes were to be examined were then immersed for 10 minutes at 37°C in 6-fold dilution of PBS in demineralized water. Incubation in this very hypotonic solution resulted in marked dispersal of the chromosomes; a similar hypotonic treatment has been used for dispersal by Chu and Giles (8). All cultures were then fixed. Fixation was usually in 4% formaldehyde in 0.075 M sodium phosphate buffer at pH 6.9 for 4-20 hours at room temperature. Formaldehyde was chosen as fixative since Stowell and Zorzoli(9) had shown it satisfactory for studying the action of ribonuclease on fixed rabbit pancreas cells, whereas they found other fixatives quite unsuitable. Carnoy's fixative (60 volumes ethyl alcohol, 30 volumes chloroform, and 10 volumes glacial acetic acid) was used for some cultures which were to be stained by the Feulgen method. Fixation in Carnoy's was for 13-40 hours at room temperature. *Digestion of fixed cells with various enzymes*: Excess formaldehyde or Carnoy's fixative was removed by thoroughly washing the fixed cultures in demineralized water before treating them with enzymes. Digestions with enzymes were at 37°C for one hour unless otherwise stated. When more than one digestion was used, slip cultures were washed with demineralized water between digestions. Control slips were incubated under same conditions in the solvent used for enzyme being studied. Unless otherwise indicated, chymotrypsin was dissolved in PBS, desoxyribonuclease in 0.2 M sodium borate buffer at pH 7.0 with 0.025 M $MgSO_4$, lipase in PBS, pancreatin in PBS, papain in PBS with L-cysteine hydrochloride at 0.020 M, ribonuclease in demineralized water, and trypsin in PBS. Enzymes were usually used at concentration of 0.25%, except for desoxyribonuclease and ribonuclease which were usually used at 0.10%. *Staining*. Slip cultures were washed in demineralized water after digestion. Those to be stained by the Feulgen method were then heated in N HCl at 60°C for 8 minutes, if they had been fixed in Carnoy's, and for 5 minutes if fixed in formaldehyde. Schiff's reagent for the Feulgen method was prepared from basic fuchsin and sodium metabisulfite according to the method of de Tomasi, [see (3)]. Staining with Schiff's reagent was for 30-60 minutes at room temperature. Staining with pyronin (commercial pyronin Y or purified pyronin) or thionin (commercial or purified) was in 0.10 M sodium acetate buffer (pH 4.9) unless otherwise noted, and for 15 minutes (occa-

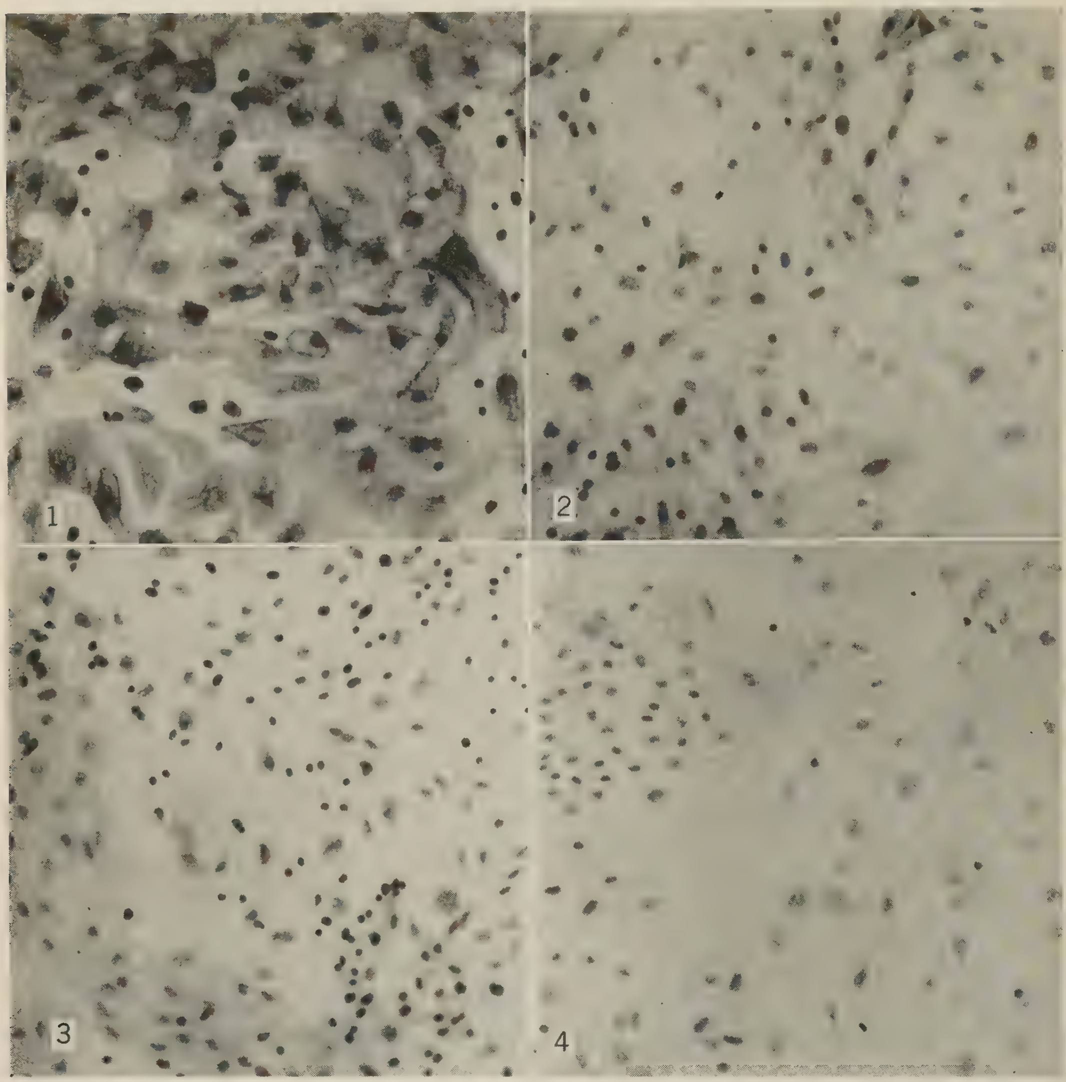

PLATE 1. Reduction in cytoplasmic pyroninophilia as result of digestion with chymotrypsin, ribonuclease, or both. All 4 monkey kidney tissue cultures had been treated with hypotonic saline and then fixed in formalin.

FIG. 1. Control culture, incubated in PBS without $CaCl_2$ and $MgCl_2$.
FIG. 2. Incubated in chymotrypsin dissolved in PBS without $CaCl_2$ and $MgCl_2$.
FIG. 3. Incubated in ribonuclease in same solvent.
FIG. 4. Incubated in a mixture of these 2 enzymes in the same solvent.

All 4 were then stained for 25 min. at room temperature in 0.06% purified pyronin in M/10 sodium acetate buffer, pH 4.2. Magnified about 180 diameters.

sionally 25 minutes) at room temperature. Pyronin Y was usually used at 0.5% but in some experiments at concentration as low as 0.06%; thionin was used at 0.1%. After staining, cultures were washed with demineralized water; they were then dehydrated, cleared, and mounted.

Results. Effects of chymotrypsin, ribonuclease, and desoxyribonuclease: The effects of treating fixed monkey kidney tissue cultures with chymotrypsin, ribonuclease, or desoxyribonuclease on some subsequent staining properties of cells are summarized in Table I. After treatment with chymotrypsin or ribonuclease, cytoplasm did not stain usual violet hue with thionin: the small amount of cyto-

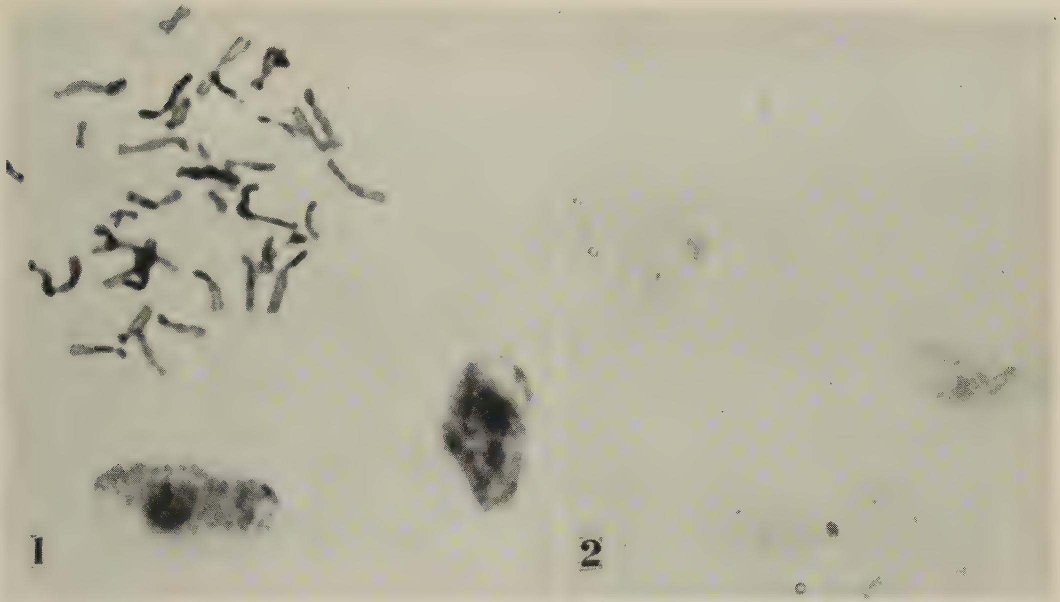

PLATE 2. Reduction in nuclear thioninophilia as a result of digestion with desoxyribonuclease. Both monkey kidney tissue cultures treated with hypotonic saline, fixed in formalin, then digested with mixture of chymotrypsin and ribonuclease in PBS without $CaCl_2$ and $MgCl_2$ to decrease interfering cytoplasmic thioninophilia.

FIG. 1. Control culture whose second incubation was in M/5 sodium borate buffer, pH 7.0, with 0.025 M $MgSO_4$.

FIG. 2. Culture had its second incubation in desoxyribonuclease dissolved in above borate buffer with $MgSO_4$.

Both were then stained 15 min. at room temperature in 0.1% unpurified thionin in M/10 sodium acetate buffer, pH 3.9. The rather vague mitotic chromosomes of Fig. 2 are clearer than those in most mitotic cells which had been treated similarly. Magnified about 2300 diameters.

plasmic staining of enzyme-treated cells was irregularly distributed and blue. When cells were treated with both chymotrypsin and ribonuclease, either simultaneously or serially, subsequent cytoplasmic staining with pyronin or thionin was decreased more than when cells had been treated only with ribonuclease; the action of this pair of enzymes reduced cytoplasmic staining with pyronin or thionin almost to zero. The effects of each enzyme alone and of the pair of enzymes on subsequent staining with pyronin are illustrated by Plate 1. The effect of desoxyribonuclease in reducing subsequent nuclear staining with thionin is shown in Plate 2.

Ribonuclease was effective when dissolved and used in demineralized water but was much less effective in either borate buffer containing $MgSO_4$ or in PBS; in PBS from which $CaCl_2$ and $MgCl_2$ had been omitted, however, ribonuclease was as effective as in demineralized water. Treatment for 15 minutes or 1 hour at 37°C with 0.10% ribonuclease in demineralized water reduced cytoplasmic pyroninophilia by large amount; reduction in cytoplasmic pyroninophilia after treatment under same conditions with 0.010% ribonuclease was slightly less.

Chymotrypsin was ineffective when dissolved and used in demineralized water but was effective in either PBS or in PBS without $CaCl_2$ and $MgCl_2$. Treatment for 1 hour at 37°C with 0.25% chymotrypsin in PBS gave a large decrease in cytoplasmic pyroninophilia; similar treatments with somewhat lower concentrations gave smaller less striking decreases.

Effects of treatment with trypsin and other treatments. Difco 1:250 trypsin had an effect similar to that of chymotrypsin; however, crystalline trypsin usually was ineffective though sometimes it gave a slight decrease in cytoplasmic pyroninophilia. In limited tests ethylene diamine tetraacetic acid, lipase, 4N HCl, pancreatin, and papain would not substitute for chymotrypsin in a decrease in cytoplasmic pyroninophilia.

Staining. Fixed cells which had been digested with ribonuclease and chymotrypsin were stained with thionin (unpurified) in M/10 sodium acetate buffer at several pH's 3.8-5.7. Within this range amount of staining was greater, the higher the pH; but the contrast between nuclear and cytoplasmic staining did not vary noticeably with pH. Similarly treated cells were stained with pyronin (unpurified) in M/10 sodium acetate buffer at pH 3.9 and 4.9. Amount of staining, especially of cytoplasm, was greater at higher pH; the contrast between nuclear and cytoplasmic staining was somewhat higher at lower pH.

Effects of chymotrypsin, ribonuclease, and desoxyribonuclease in reducing pyroninophilia were much more strikingly shown when purified pyronin was used than when unpurified pyronin was used. Even after digestion with both chymotrypsin and ribonuclease, the cytoplasm stained considerably with unpurified pyronin but much less with purified stain. The effects of these 3 enzymes in reducing thioninophilia were shown about equally clearly with unpurified thionin as with purified thionin, the hues obtained, however, were bluer with purified stain.

Chromosomes of rhesus and cynomolgus monkeys. Many mitotic cells in cultures which had undergone treatment with hypotonic PBS and had been digested with ribonuclease and chymotrypsin had their chromosomes sufficiently well spread and well stained for counting (Fig. 1 of Plate 2). Counts of some of these were made: the value of 42 chromosomes/cell was found for each species. This count for the rhesus monkey is the same as that reported by Darlington and Haque (10).

Discussion. The mechanism by which chymotrypsin decreases cytoplasmic pyroninophilia and thioninophilia of formalin-fixed monkey kidney cells is not clear. That chymotrypsin preparations were effective in this regard because of contamination with ribonuclease is not indicated since chymotrypsin was more effective in PBS than in demineralized water whereas ribonuclease was more effective in demineralized water than in PBS. It seems reasonable to assume that chymotrypsin was effective because of its proteolytic activity; but whether this proteolysis was effective (a) through hydrolyzing pyroninophilic thioninophilic protein(s), (b) through removing ribonucleic acid from cells, or (c) in some other way cannot be said. Similarity of action of chymotrypsin to that of ribonuclease in removing evenly distributed cytoplasmic violet-staining thioninophilic material but not the irregularly distributed cytoplasmic blue-staining thioninophilic material suggests that mechanism (b) above may obtain. Since crystalline trypsin was ineffective or nearly so, the chymotrypsin-like activity of Difco 1:250 trypsin may have been due to contaminating chymotrypsin.

The effect of ribonuclease in decreasing cytoplasmic pyroninophilia and thioninophilia is very probably due to its hydrolysis of cytoplasmic ribonucleic acid. Brachet(11) used this enzyme as early as 1940 for cytochemical detection of ribonucleic acid.

The effect of desoxyribonuclease in decreasing nuclear pyroninophilia and thioninophilia is very probably due to hydrolysis of desoxyribonucleic acid. The results obtained on similar cells using Feulgen stain for desoxyribonucleic acid (Table I) are not in disagreement with this interpretation. Others have obtained similar results. Catcheside and Holmes(12) reported abolition of basophilia in bean-root chromosomes with either a noncrystalline desoxyribonuclease preparation from spleen (only if used after digestion of bean cells with ribonuclease, however) or with a noncrystalline desoxyribonuclease preparation from pancreas (with or without preceding digestion with ribonuclease). Brachet and Shaver(13) found that noncrystalline desoxyribonuclease prepared from beef pancreas would abolish basophilia in chromosomes if Mg^{++} were present but not in the absence of Mg^{++}; no ribonuclease was needed. More recently, crystalline preparations of desoxyribonuclease have been found very effective in decreasing nuclear basophilia [see, *e.g.*, (4)].

The finding that unpurified pyronin revealed the actions of enzymes much less clearly than purified pyronin suggests that at least one of the contaminating reddish pigments stains cellular materials different from those stained by pyronin itself. On the other hand, the mauve contaminant in unpurified thionin appears to stain the same cellular materials as does the bluer thionin since the only noticeable effect of its removal was a shift in hues of stained cellular materials toward the blue.

Use of digestions with these and other enzymes may prove useful in cytochemical studies of virus-infected cells in tissue culture.

Summary. A large decrease in cytoplasmic pyroninophilia and thioninophilia resulted from digestion of formalin-fixed monkey kidney tissue culture with either chymotrypsin or ribonuclease. This pair of enzymes decreased cytoplasmic pyroninophilia and thioninophilia more than either enzyme alone. Digestion of similar cultures with desoxyribonuclease gave a large decrease in nuclear pyroninophilia and thioninophilia. The effects of these 3 enzymes on pyroninophilia of these cells were more clearly shown when a purified pyronin was used.

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Long-Term Culture of Tissue Cells in Magnet Stirred Fluid Suspensions.

(25013)

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One of the most important contributions to development of tissue culture technics has been the observation that tumor cells can multiply as freely suspended cell populations in fluid media(1,2). This discovery served to dispel the concept that tissue cells require anchorage to a solid substrate for multiplication, and methods were subsequently developed by Earle and co-workers(3) for first practical cultivation of animal cells on a large scale. Using 1-liter boiling flasks on Brunswick rotary shakers, these investigators successfully propagated massive fluid-suspensions of a number of cell lines and studied growth characteristics and metabolism of these free cell populations(4,5,6). There have been numerous subsequent reports on cultivation of animal cells in suspension(7-13), and other systems have been used. Preliminary experiments* indicated that tissue cells may be propagated in fluid suspensions agitated by means of a magnetic stirring bar resting on floor of culture bottles. This system has been used by us for continuous propagation of 3 cell lines required in large quantity for preparation of nucleoproteins in immunological studies with tissue culture cells(14). This method of cultivation has proven practical for the long-term culture of these cell lines and an economical means of providing large numbers of cells for various needs. Methods used and some results are reported here.

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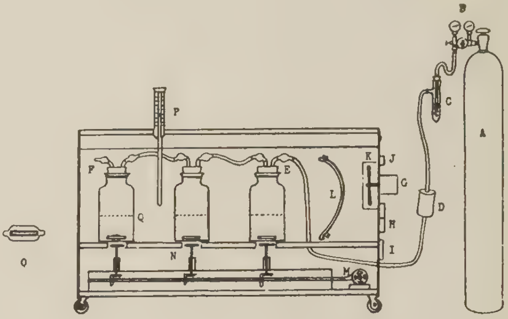
* P. A. Eichorn and A. Der Aris (unpublished data), Amer. Cyanamid Co., Pearl River, N. Y.

Materials and methods. Cell-strains and culture medium. The cell strains chosen for cultivation by these methods were the MCN line, derived from human leukemic bone marrow(15), and 2 human epidermoid carcinoma strains, H. Ep. #1(16), and HeLa(17). The culture medium was composed of either 30% calf or horse serum and 0.5% lactalbumin hydrolysate, incorporated into Earle's balanced salt solution(18) and supplemented with vitamins in concentrations reported essential for HeLa cells(19). To control bacterial and fungal infection, penicillin at 250 μ/ml, neomycin at 20 μ/ml and nystatin at 50 μ/ml were incorporated into all culture media. Culture vessels were silicone-coated,† 1-, 2-, or 9-liter Pyrex bottles, carefully selected with slightly convex bases. Each bottle contained a magnet encased in Pyrex glass (Fig. 1, 0) designed to provide smallest possible grinding surface against the convex curvature at bottom. Since an early experiment showed desirability of continuous gas exchange (Table I), all vessels were thereafter fitted with rubber stopper‡ which provided an inlet and outlet for continuous flow of 5% CO_2 in air, approximately 140 ml/hr during incubation. Culture bottles were incubated at 37°C in portable incubator units designed for magnet stirring (Fig. 1). Briefly, the unit consists of portable wooden box insulated with 1-inch Celotex wallboard which serves as incubator. Heat is provided by a DeKhotinsky thermoregulator and an Allen-Bradley automatic starter. The magnet stirring assembly is mounted in a compartment beneath the incubator and consists of 3 metal rotating holders for three 2½ x 2½ inch magnets, each posi-



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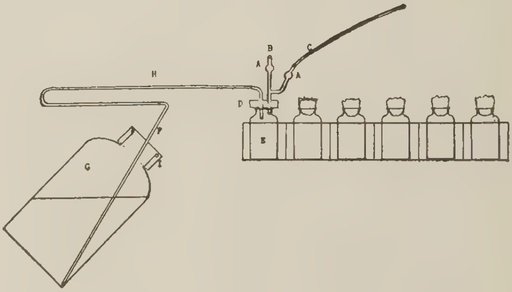

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FIG. 1. Diagram of incubator and magnet stirring unit. A, Tank of 5% CO_2 in air; B, high and low pressure gauge (Matherson Co., East Rutherford, N. J.); C, bubbler tube containing Carbowax 400 (polyethylene glycal) (Carbide & Carbon Chemical Co., N. Y.); D, air purifier and flow equalizer (Koby Corp., Boston); E, cotton-packed gas inlet of culture bottle; F, cotton-packed gas outlet of culture bottle; G, electric motor (1750 r.p.m.) for fan; H, DeKhotinsky thermo-regulator (Central Scientific Co., Chicago, Ill.); I, Allen-Bradley automatic starter (Allen-Bradley Co., Milwaukee, Wis.); J, pilot light; K, 10-in. fan and nichrome heating coil; L, insulated panel to circulate heat over culture bottles; M, Bodine electric motor with changeable gears for 200, 250 and 300 r.p.m.; N, adjustable magnet holders; O, magnet encased in Pyrex glass; P, thermometer; Q, the level of medium.

FIG. 2. Apparatus for aseptic transfer of cell suspensions to centrifuge tubes. A, Cotton-packed filter bulbs; B, finger tip control; C, rubber tubing to vacuum line; D, rubber stopper fitted with glass tubing connections from culture bottle to vacuum line. (The cell suspension is transferred to the centrifuge bottle by placing the stopper securely onto the neck of the centrifuge bottle and filling the bottle to the desired level by use of vacuum and fingertip control "B."); E, centrifuge bottles; F, glass tubing to culture bottle; G, culture bottle; H, rubber tubing; I, protective cover with small hole for entry of glass tubing.

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† General Electric, SC-87, Silicone Products Dept., Waterford, N. Y.

‡ Cafe Au Lait rubber, Baxter Rubber Co., Newark, N. J.

TABLE I. Effect of Gas Flow on Multiplication of H.Ep. #1 Cells in Magnet Stirred Fluid Suspension Cultures.

Culture #	Gas flow during incubation	No. of nuclei/ml						
		0 days	3 days	7 days	10 days	14 days	17 days	21 days
		× 1000						
1	None, cultures sealed	300	326	333	520	346* 173	275	331
2		”	410	533	933	489* 245	300	410
3		”	250	510	553	379* 189	210	280
4	5% CO_2 in air, 140 ml/hr	300	400	1,973	3,950	3,306* 1,653	4,150* 2,075	4,210
5		”	280	1,773	3,224	2,457* 1,228	3,600* 1,800	3,822
6		”	405	1,670	3,191	2,580* 1,290	3,200* 1,600	3,502

* First figure represents No. of nuclei/ml of culture medium before half the cells were harvested.

tioned directly beneath glass-covered opening in floor of incubator. Holders for magnets are mounted on Monel metal gear system and rotated at 200 rpm by an electric gear-reduction motor. *Planting of cultures, culture medium changes and cell harvests.* One- and 2-liter bottles were generally seeded with 100-300 ml of cell suspension prepared with aliquot of cells scraped from glass floor of stock strain culture flasks (modified T-60 flasks of Earle and Highhouse) (20). These cells were resuspended in culture medium at 300,000 cells/ml with approximate error of ± 30,000 cells(21). At consecutive culture medium changes, the volume of medium was increased slowly to provide a maximum of 300-400 ml in each one-liter bottle and 600-800 in each 2-liter bottle. When no further increase in cell concentrations occurred in the maximum volume of medium, either one-half of cell population was removed, or the entire cell population was used as seed for a 9-liter culture bottle. The 9-liter bottles were seeded with not less than 300,000 cells/ml in one liter of medium. This volume was increased at subsequent culture medium changes to a maximum volume of 3 liters/bottle. The culture medium was changed twice weekly, *i.e.*, once each 3-4 days. At this time, cell suspensions were removed from bottles, collected in 250 ml centrifuge tubes and sedimented in an International #2 centrifuge at 600 rpm for 5 minutes. A diagrammatic sketch of the apparatus, operated by vacuum, which permits aseptic transfer of cell suspensions to centrifuge tubes by one operator, is shown in Fig. 2. Following centrifugation the supernatant was removed and discarded. The packed cells were taken up in a volume of fresh medium, pipetted back and forth through tip of a 10 ml serological pipette to disperse clumping resulting from centrifugation and returned finally to their respective culture bottles. The bottles were filled with desired volumes of fresh medium, stoppered, and placed again over a rotating magnet at 37°C. When cells were to be collected from cultures, the gently packed cells resulting from centrifugation were resuspended in a given volume of medium and a measured portion of this suspension returned to original culture bottle. At time of medium changes and collection of cells, cell nuclei counts were carried out as a measure of cell numbers in each culture. This was accomplished by aseptic removal of a 5-ml sample from each culture which was agitated over a small "Mag-Mix"§ to insure removal of a representative sample. Cell nuclei were prepared and enumerated by the method of Sanford *et al.*(21).

Results. When culture bottles were initially seeded and no cells collected at subsequent culture medium changes, the following phases in the life of cell population were observed for all 3 cell strains: a) an initial lag phase of 2-3 days, b) a phase of active cell multiplication reaching maximum concentrations around 9-10 days and c) a phase beginning around 12 days consisting of no further increase in cell numbers and characterized by presence of many dead cells. These results appear generally to coincide with those obtained by Kuchler and Merchant with L strain cell suspensions in shaker cultures(8). It was soon noted that highest yields of cells could be obtained by collecting one-half of the cells from populations in phase (b) and repeating this each 3-4 days or semi-weekly. Comparative cell yields of cultures from which cells were collected weekly and semi-weekly revealed that although one-half of the cells are removed from these cultures each 3-4 days, maximum cell populations are almost regularly attained at end of this period. One 9-liter bottle culture of MCN cells, when one-half of its cells were harvested each 3-4 days, yielded an average of 21.5 ml of centrifuge-packed cells each week for 7 months of continuous cultivation. Maximum cell concentrations in these cultures ranged from 3-5 million cells/ml of culture medium.

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§ Magnetic motor stirrer, Catalogue #18852, Central Scientific Co., Chicago, Ill.

In one experiment, an attempt was made to study variation of multiplication of cells suspended freely in fluid medium with and without continuous gas exchange. For this study H. Ep. #1 cells harvested from one 9-liter culture bottle were used as an inocula for 6 one-liter bottles, each containing 300 ml of medium. Subsequently 3 of these bottles were sealed with rubber stoppers and 3 were closed with stoppers which provided an inlet and outlet for a continuous flow of gas during incubation. Medium changes or cell harvests were made semi-weekly; each culture was sampled at this time for cell nuclei counts (Table I).

These results show an initial lag in all cultures of about 3 days, after which a phase of active cell multiplication began. Maximum cell concentrations were reached at about 10 days. Rate of gas exchange appears all-important to cultures which under these conditions are restricted to a renewal of culture medium each 3-4 days. These observations appear to conform with those of Earle *et al.*(3), who found that sealed shaker cultures which were flushed out with 5% CO_2 in air only each 2-3 days exhibited evidence of cell death from lack of oxygen and from acidity.

Discussion. Cells derived from human leukemic bone marrow (MCN) remained freely dispersed in these cultures, exhibiting little evidence of clumping. However, HeLa cells and to a lesser extent, H. Ep. #1 cells, tended to form spherical aggregates of less than 1 mm, such as that described by Earle *et al.* in shaker cultures(3). These aggregates appeared healthy, whereas many of the singly occurring cells appeared fragmented and full of cytoplasmic granules. Often, cells could be seen adhering to inside walls of culture bottle, forming a ring where medium swirled over them. The collection of cells at this site was not excessive, however, and could generally be dislodged by gentle shaking of culture at room temperature.

Earle *et al.*(3) reported extensive injury to cells when using a cylindrical magnetic stirrer which rolled over a flat bottom culture container. Little evidence of such destruction was noted with the methods here described, nor of protein thread-precipitate from culture medium which sometimes has been observed in shaker cultures. No foaming of culture medium was observed at rate of agitation used.

Cells collected from these cultures have on occasion been transferred to stationary culture flasks. When this is done, the viable cell clumps attach themselves to the glass, leaving broken cell fragments and debris floating free in supernatant medium. This debris can be removed upon the first change of medium; such cultures require one or 2 passages, with use of trypsin for detaching cells from glass, before the clumps become evenly dispersed and uniform cell sheets are obtained.

Summary. Human leukemic bone-marrow-derived (MCN) cells and human epidermoid carcinoma cells, (HeLa) and (H. Ep #1), have been continuously cultured over 1 year as suspended cell populations in a magnet-stirred fluid medium. This method of cultivation is a practical means of providing large numbers of freely suspended tissue cells for immunochemical and other studies. Cell mul-

tiplication for all 3 strains studied is characterized by: a) an initial lag phase of 2-3 days, b) a phase of active cell multiplication, reaching maximum concentrations around 9-10 days, and c) a phase beginning around 12 days with no further increase in cell numbers. Under conditions described, maximum cell yields were obtained by collecting one-half the cells from populations in phase (b) each 3-4 days.

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Radioautographic Study of Interkinetic Nuclear Migration in the Neural Tube.* (25014)

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Mitotic figures in the early neural tube occur only adjacent to central canal. This led to the view that cells near the lumen constitute a distinct type, which alone divide, and thus serve as "mother cells" for the entire wall. However, histological studies(1,2) indicated a migration of nuclei in the neural tube, *i.e.*, after division at luminal margin, a nucleus moves into the depths of wall during interkinetic period, and returns to the free surface as it passes into the prophase of the next division. Nevertheless, a recent discussion(3) continues to favor the long held germinal cell theory. The response of the early neural tube to colchicine(4,5) gave the first experimental confirmation of the migration concept. Additional evidence came from measurements of amount of deoxyribonucleic acid (DNA) in cells of neural tube. Sauer and Chittenden(6) found that mean amount of DNA in nuclei at some distance from the lumen considerably exceeded the 2n value. Since synthesis of DNA precedes each cell division during duplication of chromosomes, this indicated that deep nuclei undergo changes preliminary to division, as the concept of migration requires. A demonstration of the site of formation of DNA in the early neural tube with tritium-labeled thymidine, known to be incorporated only into new DNA,

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* This investigation supported by research grants from NINDB and Dental Institute, NIHPHS.

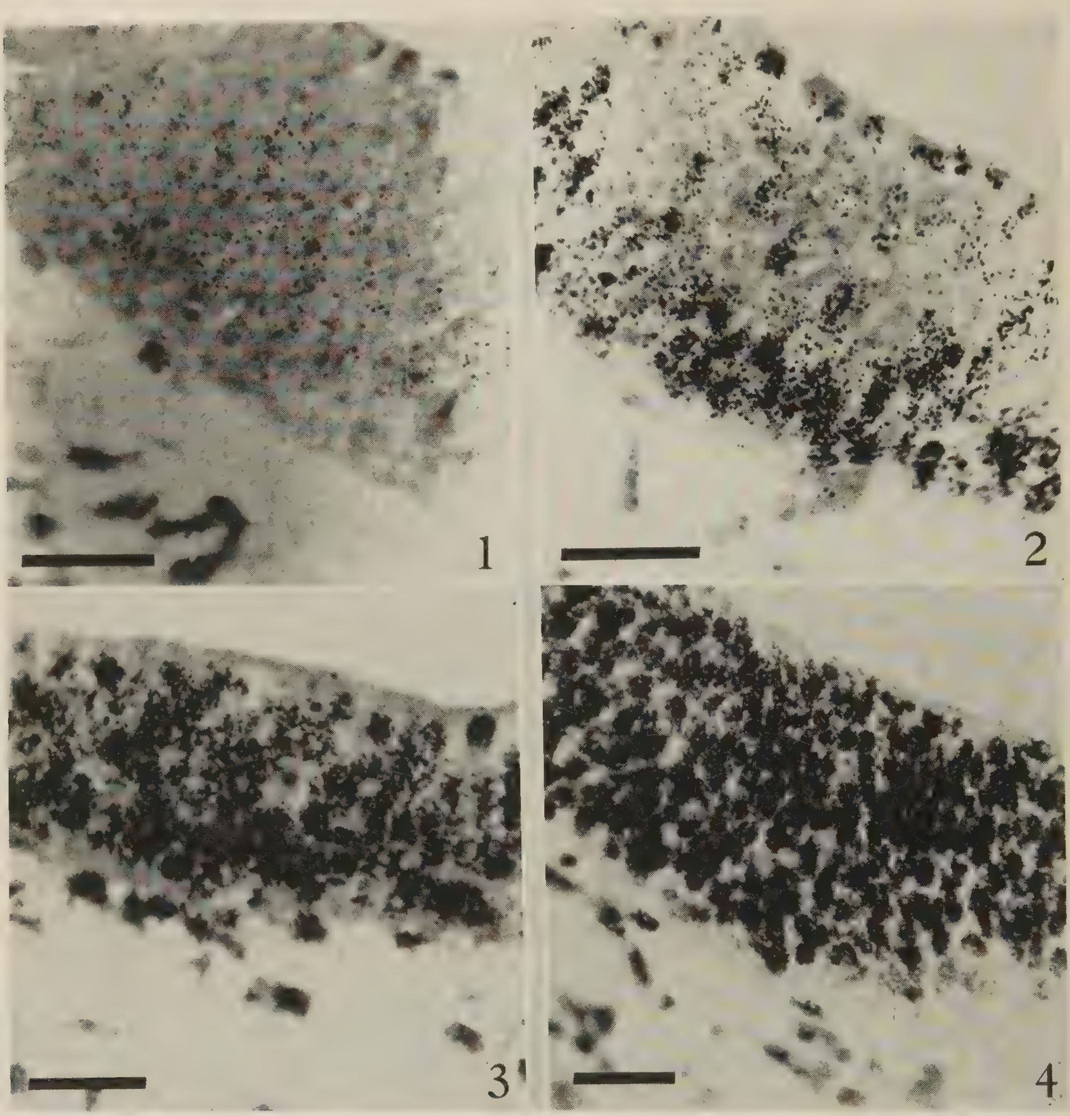

FIG. 1-4. Radioautographs of neural tube (brain) of chick embryos fixed at varying intervals after administration of thymidine-H^3. Lumen at top. Exposed 15-16 days. Hematoxylin. Line indicates 25 μ. FIG. 1. 1 hr, stage 16 embryo. FIG. 2. 4 hr, stage 18. FIG. 3. 8 hr, stage 16. FIG. 4. 12 hr, stage 17.

should provide a graphic test of the migration concept. The present communication enlarges upon a preliminary report(7) of such a study in the neural tube of the chick embryo.

Procedure. Eggs containing 2½ day old embryos were opened by the Harkmark-Graham(8) method. Thymidine-H^3 was dropped onto each embryo (dosage/embryo, 50 μc in .05 ml). Further incubation time ranged from 1 to 24 hours, giving embryos of stages 16 to 19(9). These were fixed in Serra's fluid, embedded in paraffin and sectioned at 6 μ. Sections were processed for radioautography by the dipping method of Messier and Leblond(10), using NTB3 emulsion (courtesy Eastman Kodak Co.), and developed after exposure times ranging from 15 to 112 days.

Results. The descriptions below apply to radioautographs of the neural tube. Time intervals refer to the interval between administration of thymidine-H^3 and fixation of the

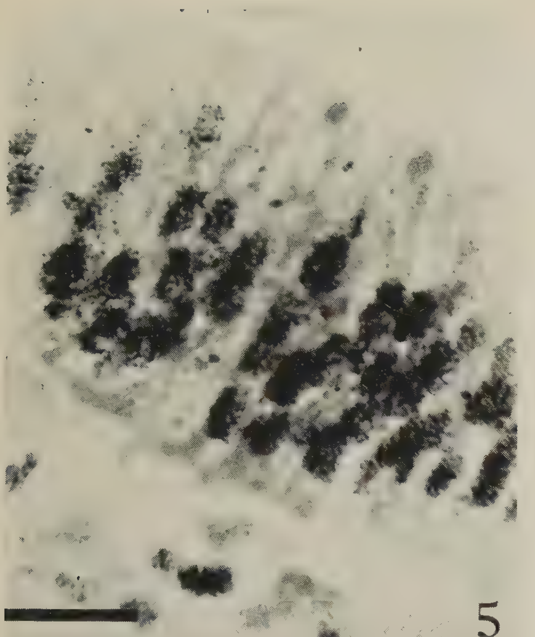

FIG. 5. Neural tube from caudal half of a stage 16 embryo. Lumen at top. 2 hr after treatment. Exposed 112 days. Aqueous basic fuchsin. Line, 25 μ.

embryo. Exposures for the radioautographs illustrated in Figs. 1-4 were all of the same duration (15-16 days); that in Fig. 5 was much longer (112 days).

1 and 2 hour intervals. These intervals are characterized by radioactivity distributed uniformly in the outer half to two-thirds of the wall, and a lack of radioactivity at the luminal margin. The labeling is light (Fig. 1) as compared to similarly exposed radioautographs of the longer time interval embryos. In order to obtain a denser radioautograph for comparison, a prolonged exposure (112 days) was made of sections from a 2 hour interval embryo (Fig. 5). Although radioactivity increases between the 1 and 2 hour intervals, the marked difference between Figs. 1 (1 hr) and 5 (2 hr) is a matter of exposure. Prolonged exposure may bring out light labeling in the inner third of the wall or involve an occasional metaphase at the lumen; however, the general distribution of radioactivity is the same, regardless of length of exposure.

4 hour interval (Fig. 2). Radioactivity extends throughout the wall, but is not distributed uniformly. A layer of greater density is located at the periphery. Although the pattern in the neural tube varies somewhat with level and age, a dark band in the outer third or fourth of the wall usually characterizes this period. The area between the dense peripheral zone and the lumen contains many lightly radioactive nuclei and a few heavily labeled nuclei. Mitotic figures at the lumen are now radioactive.

8 hour interval (Fig. 3). Strongly radioactive nuclei extend uniformly from periphery to lumen.

10, 12, 16 and 24 hour intervals (Fig. 4). These show no change beyond the previous interval, other than a progressive increase in density up to 16 hours, when practically all nuclei are labeled. The 24 hour interval embryo resembles the 16 hour.

Discussion. The results obtained in the early neural tube with thymidine-H^3 clearly verify the concept of an interkinetic migration of nuclei(1,2). Radioautographs of the 1 and 2 hour interval embryos demonstrate that only nuclei in the peripheral region of the tube, away from the lumen, synthesize DNA. Therefore, new nuclei produced by mitosis at the lumen must move away from the lumen and enter the peripheral zone before synthesizing any significant quantity of DNA. Nuclei remain in the peripheral zone, while synthesizing DNA, for at least 4 hours, since the most radioactive nuclei of the 4 hour interval embryos occur in this zone. The nuclei then return to the luminal border to divide. Some nuclei are already part way through their synthetic period at the time of application of the isotope, and, therefore, incorporate less than the maximum quantity of thymidine-H^3. In the 4 hour interval radioautographs these are the weakly radioactive nuclei of the juxtaluminal zone. Since this zone also contains a few strongly radioactive nuclei, probably 4 hours is almost long enough for a complete period of synthesis of DNA.

At 8 hours, heavily labeled nuclei are moving both to and from the lumen. Consequently, the layering effect does not appear at this time interval, or at later intervals. However, the density of the radioautographs does increase with time, indicating cumulative incorporation of thymidine-H^3 during subsequent periods of

DNA synthesis. The continued availability of thymidine-H^3 to the chick embryo from a single application is in contrast to its fate in mammals, where it is rapidly metabolized, and therefore labels nuclei over only a short period of time(11). A preliminary report(12) of work similar to the present study illustrates this species difference, while confirming nuclear migration in the neural tube.

It should be emphasized that the fundamental concept involved here is not that of nuclear migration in itself, but rather a recognition of the structure of the early neural tube as a columnar epithelium(13,14). Nuclear migration is a necessary accompaniment to the rounding up of the cell in mitosis and its subsequent elongation. The cell maintains its firm terminal bar attachment at the lumen as long as it is an element of a columnar epithelium, hence, the nucleus is drawn toward the lumen as the cell rounds up in preparation for division. Following mitosis, the nucleus is carried peripheralward as the cell elongates to renew its attachment to the outer limiting membrane.

Conclusions. Radioautographs of early neural tube of chick embryos treated with thymidine-H^3 for varying intervals of time give evidence substantiating the concept of interkinetic migration of nuclei. Synthesis of DNA occurs only in nuclei of peripheral part of wall, and does not take place in those of juxta-luminal zone. The period of DNA synthesis continues for at least 4 hours, but does not extend much beyond this time.

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Serum Adenosine Deaminase Activity in Cancer.*† (25015)

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Straub and his associates(1) reported that adenosine deaminase, the enzyme that catalyzes the deamination of adenosine to inosine, was present in plasma and was elevated in 92% of the 527 cancer patients studied. A similar frequency was reported by Letnansky and Seelich(2). Such a high frequency of elevated serum or plasma enzyme activity indicated possible diagnostic utility. Our previous studies on other serum enzyme activities in patients with cancer(3,4,5) showed that elevations in these activities were related more directly to growth or activity of tumor, rather than to its presence. It was accordingly decided to determine, first, activity of the serum adenosine deaminase in groups of normal persons and patients with

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TABLE I. Eight Patients with Cancer Who Showed Elevated Serum Adenosine Deaminase Activity at First Determination.

Diagnosis	Serum deaminase activity (units)
Reticulum cell carcinoma	16.2
Ca breast	21.7
Reticulum cell carcinoma	44.0
Lymphosarcoma	25.2
Ca breast	17.4
Ca ovary	20.6
Ca breast	28.2
Reticulum cell carcinoma	38.6

cancer. Secondly, activities of this enzyme were determined sequentially and at frequent intervals during periods of one to 2 months in 4 patients with cancer and were correlated with clinical, biochemical or other evidence of growth or regression of tumor. Since similar features were evident in these 4 cases, detailed data are presented for only one case.

Materials and methods. The method employed was essentially that described by Straub and his associates(1). This is based on the release of ammonia effected in 1 hr at 37° by 1 ml of serum in a reaction mixture containing 0.9 ml of 0.014 M adenosine, 0.1 ml of 1.33 M phosphate buffer of pH 7.8 and 2 ml of serum. The released NH_3 was determined by nesslerization. Adenosine was obtained from Pabst Laboratories. One unit of activity was defined as equal to 1 μg of NH_3-N liberated under the stated conditions.

Results. Values in normal persons. The adenosine deaminase activity did not decrease in sera kept in the refrigerator or deep-freeze for at least one month. Mean value for this enzyme activity in 14 normal persons was 10.5 μg of ammonia N liberated/ml of serum in 1 hour (10.5 units). Standard deviation was 2.1 μg of ammonia N (2.1 units); and 2.5 standard deviations about the mean were used to designate the normal range, 5.3 to 15.8 μg of NH_3-N. These results are essentially in agreement with those obtained by Straub and his coworkers(1).

Serum deaminase activity in patients with cancer. The serum deaminase activity was elevated upon the first determination in 8 of 55 patients with various types of cancer (Table I). This frequency, 15%, was much less than that reported by Straub(1) or Letnansky(2). This low incidence is similar to those obtained for other serum enzymes in cases of proven cancer(6,7) and raises the possibility that, as for these other enzymes, serum deaminase elevations are related more specifically to activity or growth of tumor. The following case illustrates this feature.

The patient was a 46 year old woman who was diagnosed as having carcinoma of the breast in 1950. Mastectomy was carried out at this time. Three years later, skeletal metastases became evident, and castration by irradiation was performed. She received testosterone and prednisone. She was readmitted to the hospital on July 31, 1958 because of increasing pain in her left thorax. At this time she showed widespread osteolytic metastases, several nodules on the skull, a liver enlarged to 2 cm below the costal margin, elevated bilirubin concentrations, 1.2 and 1.9 mg per 100 cc, a bromsulfalein retention of 40% and an alkaline phosphatase level of 8.5 Bodansky units. Between Aug. 1 and Aug. 12, the daily urinary calcium excretions were above normal, ranging from 248 mg to 763 mg. The serum calcium was elevated to 12.4 mg per 100 cc on Aug. 1. On Aug. 6, administration of an actinomycin was begun at an intravenous dose of 1 mg per day, was stopped on Aug. 20 because of a low platelet count (42,000) and reinstituted on Sept. 9.

Serum deaminase determinations were begun on Aug. 12 (Table II). Increases of urinary calcium excretion and serum calcium levels on about Aug. 25 and elevations in bilirubin about Sept. 2, evidences of increasing growth of tumor in skeleton(8) and liver(3), were paralleled by increases in serum deaminase activities and in worsening of patient's clinical condition.

Discussion. Although earlier investigators determined the adenosine deaminase activity in plasma, Letnansky and Seelich(9) showed that there was no difference in activity between the plasma and serum of the same specimen. The low incidence of serum deaminase elevations obtained in our study would appear to be due to the fact that the group of patients in our study had less advanced neoplastic disease. Letnansky and Seelich(9) reported that other serious illnesses such as

TABLE II. Parallelism between Rise in Serum Deaminase Activity and Manifestations of Growth of Metastatic Carcinoma of Breast.

Date	Clinical status	Bilirubin, mg/100 ml	Serum Ca, mg/100 ml	Urinary Ca excretion, mg/24 hr	Serum deaminase activity, units
8-12	Widespread osteolytic metastases, skull nodules, slightly enlarged liver, some nausea, some pain	2.39		525	21.8
-13	Some improvement, regression of skull nodules		10.9	525	
-15			11.2		19.5
-18			12.1	241	22.2
-19	Abdominal girth greatly increased, edema	2.88		241	21.0
-20	Possible drug toxicity, otherwise condition about the same		11.2	259	20.4
		3.18	11.2	312	18.2
-25			11.5	476	19.5
-26		2.73		476	21.8
-29			13.0	539	
-31			14.1	557	
9- 2	Downhill course; increasing weakness, abdominal distention and disorientation	5.56	12.6	471	26.7
-10		21.20	13.4		28.5

tuberculosis and pneumonia yielded a high incidence of elevations in adenosine deaminase activities. This finding is similar to that obtained for other serum enzymes which are not specific but are also elevated in grave diseases involving tissue damage or destruction (10). The parallelism between increasing serum deaminase activity and evidence of increase in tumor growth in the patient described above supports this conclusion.

Summary. Serum deaminase activity was elevated in 15% of a group of 55 patients with proven cancer. Extent of elevation was shown to parallel tumor growth and clinical condition of patient. These findings are similar to those obtained for other serum enzymes.

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Effects of Reserpine and Serotonin on Milk Secretion and Mammary Growth in the Rat.* (25016)

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Reserpine can induce mammary growth and initiate lactation in the estrogen-primed rabbit(1), and increase prolactin content of rabbit pituitary(2). Reserpine has also been observed occasionally to elicit breast growth and lactation in women(3). Attempts by us to induce mammary growth and milk secretion in rats with reserpine have been unsuccessful, although reserpine prolonged mammary secretion in postpartum rats after litter removal(4, 5). Recently we reported that epinephrine and acetylcholine produced mammary growth and milk secretion in the rat following estrogen administration, but were ineffective when given alone(6). It was of interest, therefore, to see whether reserpine could similarly induce mammary development and secretion in the estrogen-primed rat. The effects of serotonin on mammary function were also determined, since this substance is released from the brain following reserpine administration(7). It was also of interest to compare both these drugs for their ability to retard mammary involution after litter removal in postpartum rats.

Methods. Mature female albino rats (Carworth) weighing 220-250 g were divided into uniform groups of 5 or 10 each and treated according to regimen in Table I. Virgin rats were injected subcutaneously once daily in 0.1 cc volumes as follows: saline, 0.9% solution; estradiol,‡ 10 μg; reserpine,§ 25 μg; serotonin creatinine sulfate, 0.5 mg serotonin base/kg BW. In postpartum rats the litters were removed on 4th day of lactation, and the mothers were injected subcutaneously for 10 days with the following: saline, 0.9% solution; reserpine, 10 or 20 μg; serotonin creatinine sulfate, 0.5 mg serotonin base/kg BW. All rats were killed on day after last injection, and the right and left inguinal mammary glands were removed for histological examination. Mammary glands were fixed in Bouin's fluid, sectioned at 6 μ and stained with eosin and hematoxylin. Lactation ratings of 0 to 7 were assigned to each gland, depending on amount of secretion observed microscopically. Percentage of parenchymal as distinct from stromal mammary tissue was estimated from histological sections. From most groups, ovaries, uterus, adrenals, and thymus glands were removed, trimmed, and weighed on Roller-Smith balance.

Results. Table I. The mammary glands of virgin rats given saline had only bare ducts, while estradiol induced limited lobuloalveolar growth which regressed to mostly ducts when followed by saline. No effects were noted with reserpine alone, while estradiol and reserpine together elicited more lobuloalveolar growth than estradiol alone. When estradiol was followed by reserpine, moderate lactation was initiated. Serotonin alone was ineffective, but increased mammary growth when given together with estradiol, and induced marked secretion and mammary enlargement when injected after estrogen. In the non-suckled postpartum rats, reserpine or serotonin partially maintained lactation and retarded mammary involution. Neither drug depressed body growth nor significantly altered weights of ovaries, uterus, adrenals or thymus. Representative mammary sections are shown in Fig. 1-6.

Discussion. These data indicate that estrogen priming is essential for reserpine and serotonin to induce milk secretion and mammary growth in the rat. These drugs were more effective when given after than when given together with estradiol. In virgin rats,

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‡ The estradiol was kindly furnished by Dr. J. O. Reed of Foundation Labs., N. Y. City.

§ We are indebted to Dr. Russel Kraay of Eli Lilly and Co., Indianapolis, Ind., and to Dr. Robert Gaunt of Ciba Pharmaceutical Products, Summit, N. J., for reserpine.

TABLE I. Effects of Reserpine and Serotonin on Mammary Growth and Milk Secretion in 14 Groups of Rats.

Total rats	Treatment*	No. rats with secretion	Avg secretion rating	Avg % mam. parenchyma
	Virgin rats			
10	Saline, 15 d	0	0	7
"	Est, 10 d	0	0	18
"	Est, 10 d Saline, 5 d	0	0	12
10	Res, 21 d	0	0	8
5	Est + Res, 10 d	1	<1	35
5	Est, 10 d Res, 5 d	4	3	45
5	Ser, 21 d	0	0	7
5	Castrate + ser, 21 d	0	0	5
5	Est + Ser, 10 d	2	<1	26
10	Est, 10 d Ser, 5 d	10	>5	57
	Postpartum rats, litters removed on 4th day			
5	Saline, 10 d	0	0	11
5	Res (10 μg) 10 d	4	2	21
5	Res (20 μg) 10 d	4	2	25
5	Ser, 10 d	2	<1	23

* Est = Estradiol. Res = Reserpine. Ser = Serotonin.

estradiol alone induced lobuloalveolar mammary growth, presumably by stimulating ovarian luteal function. Estrogens also increase prolactin content of the rat pituitary, but do not initiate mammary secretion unless ovaries are removed(8). In these experiments the mammary glands regressed following cessation of estradiol injections, but subsequent administration of reserpine or serotonin for 5 days apparently released sufficient amounts of prolactin and perhaps other hormones to initiate milk secretion and maintain the mammary lobuloalveolar system. Apparent increase in mammary parenchymal tissue can be partially attributed to filling of alveoli and ducts with secretion. Ability of these drugs to prolong mammary function in postpartum rats following litter removal is believed to depend on a similar release of prolactin and perhaps other hormones from the pituitary.

The pathways through which reserpine and serotonin exerted their effects on the mammary gland remain to be determined. Since the hypothalamic portion of brain has been shown to influence secretion of several anterior pituitary hormones(9), it is possible that these drugs activate stimulating or inhibiting centers in the hypothalamus which induce release of pituitary prolactin. There is evidence that the hypothalamus may inhibit secretion of prolactin(10), and since reserpine has been reported to depress certain hypothalamic functions(11), this would remove the inhibition to prolactin secretion. Serotonin may also be involved in this process, since reserpine markedly reduces serotonin levels in the brain (7). There is no evidence that reserpine or serotonin directly influence the anterior pituitary or mammary gland, although this has not been investigated. The effects of these drugs apparently were not mediated through ovaries or adrenals, since dose levels employed did not significantly alter weights of these organs or of uterus and thymus. The possibility cannot be excluded however, that these drugs induced transitory changes in secretion rates by ovaries and adrenals.

Previously only 2 stimuli have been shown capable of initiating lactation and increasing pituitary prolactin secretion, namely certain steroids, particularly estrogens, and the nervous stimulus of suckling or milking(8). We have now demonstrated that 3 other natural body substances, *viz.*, epinephrine, acetylcholine and serotonin, can also initiate mammary secretion. Preliminary evidence suggests that they increase prolactin synthesis and/or release, perhaps through a common mediator in the hypothalamus where they are normally present in high concentrations(9). The lactation-stimulating action of estrogens or suckling may also be mediated through one or more of these neurohormones.

Summary. 1. In mature virgin rats, daily subcutaneous injections of 20 μg reserpine or .5 mg serotonin creatinine sulfate base/kg BW for 5 days, following daily injections of 10 μg estradiol for 10 days, initiated milk secretion in 14 out of 15 rats and induced growth of mammary lobuloalveolar tissue. When either drug was injected with estradiol for 10 days, mammary growth was greater

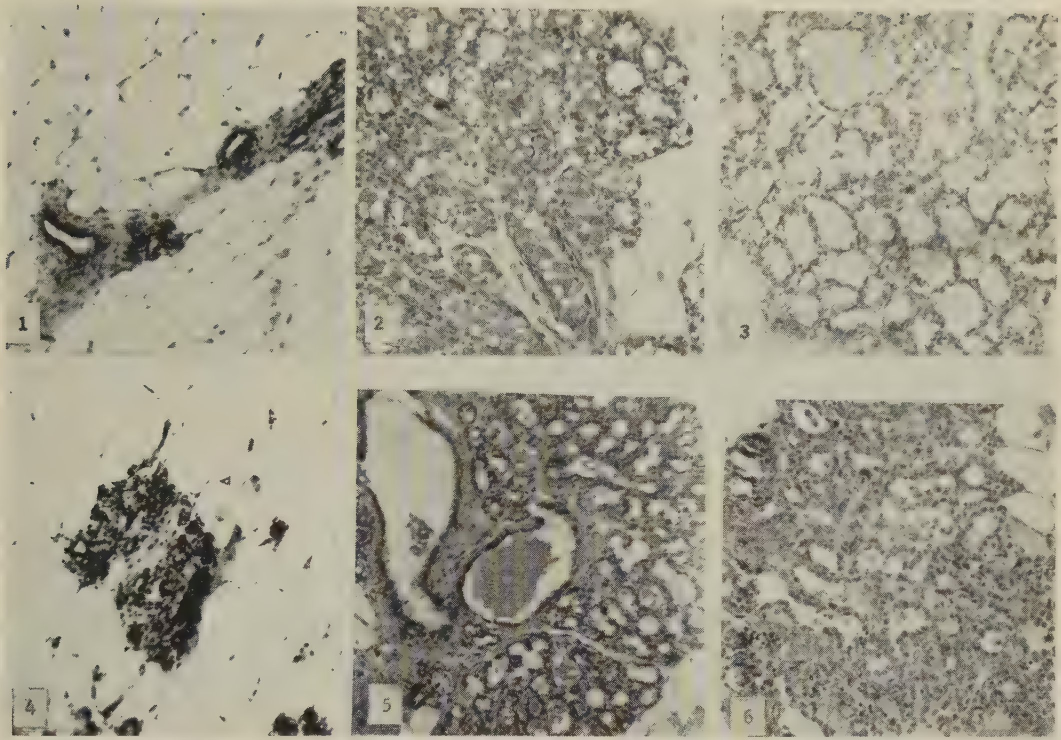

FIG. 1-3. Mammary sections from virgin rats: (1) estradiol 10 d, saline 5 d; (2) estradiol 10 d, reserpine 5 d; (3) estradiol 10 d, serotonin 5 d.

FIG. 4-6. From postpartum rats 10 d after litter removal: (4) saline, (5) reserpine, (6) serotonin. × 67.

than with estradiol alone, but there was little evidence of milk secretion. Injections of reserpine or serotonin alone for 21 days failed to elicit mammary growth or secretion. 2. In rats following litter removal on 4th day after parturition, milk secretion ceased and mammary glands showed pronounced involution 10 days later. Injections of 10 or 20 μg reserpine or .5 mg serotonin creatinine sulfate base for 10 days following litter removal, retarded mammary involution and maintained secretory function. 3. It is concluded that in estrogen-primed virgin rats or in post-partum rats after litter removal, reserpine and serotonin stimulate secretion of prolactin and perhaps other factors by the pituitary favorable to mammary growth and lactation.

ADDENDUM: Preliminary assays of pituitary prolactin content of rats indicate that epinephrine, acetylcholine and probably reserpine and serotonin induce a discharge of prolactin from the pituitary shortly after injection of these drugs.

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Abnormal Serum Protein Observed in Trypan Blue-Treated Rats.* (25017)

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Trypan blue has been known to bind with albumin and also with α-globulin when the quantity of dye is sufficient(1,2). It is also known that thyroxine binds with specific serum protein which has an electrophoretic mobility between that of α-1 and α-2 globulin at pH 8.6(3,4). Recently Crispell *et al.*(5) reported an *in vitro* experiment indicating that trypan blue competes with thyroxine for binding sites on the "thyroxine-binding protein," which thus would make more thyroxine available to the cells because of the weaker binding capacity of the secondary carrier. While investigating this problem, it was found incidentally that the electrophoretic pattern of the serum obtained from trypan blue-treated animals differed from that of control animals. This fact is of particular interest in considering protein metabolism under these conditions and indicates there might be synthesis of an abnormal protein in response to trypan blue treatment. This paper presents the results of a preliminary study of this abnormal protein component of serum.

Materials and methods. Seventy-nine male Holtzman rats, weighing 180-250 g, were used. Four ml of 0.5% saline solution of trypan blue were injected intraperitoneally once or twice daily for up to 6 days. Blood was taken at various intervals after the last injection of trypan blue. Serum was developed by the standard paper-electrophoretic method in a Spinco apparatus. Boric buffer (pH 7.5), barbital buffer (pH 8.6) and boric-borax buffer (pH 9.2) were used. The paper strip was stained with Spinco Dye B-1. Density of the strips was measured in a Spinco Analytrol to determine relative concentration of each component.

Results. An abnormal band between α-1 and α-2 globulin was consistently found in all trypan blue treated animals (Fig. 1). The abnormal band appeared during electrophoresis at pH 8.6 and 9.2, while it did not separate from α-2 globulin at pH 7.5, though its presence was apparent from dense staining in a portion of the α-2 globulin when compared to normal serum. The best separation was obtained at pH 9.2, the abnormal protein lying midway between α-1 and α-2 globulin. Trypan blue itself stayed at the origin with all 3 variations of pH. This probably indicates that trypan blue is absorbed by the paper rather than that trypan blue remained unbound to protein in the serum since the dye was precipitated almost completely from serum by 0.5% trichloroacetic acid, leaving a clear supernate. The well known contaminant red dye in trypan blue migrated only a short distance on the paper.

The abnormal protein band could be detected in the serum 1, 6, 12, and 24 hours after a single injection of trypan blue *in vivo*. Its disappearance from the blood was not studied extensively, but on one occasion it was still present 5 days after a single injection of trypan blue. This component was present in a greater amount than was α-2 globulin

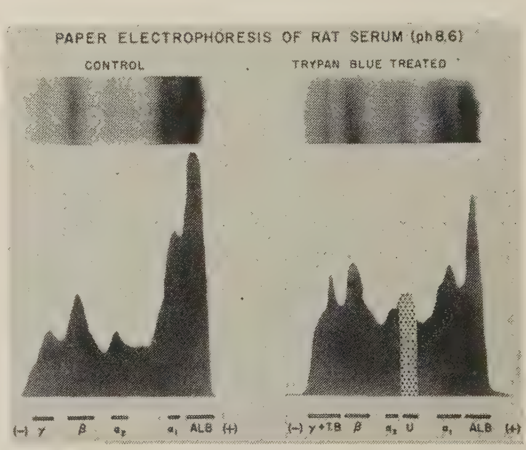

FIG. 1. In trypan blue-treated animal, 4 ml of 0.5% trypan blue in physiological saline were inj. intraper.; in the control, 4 ml physiological saline were inj. in same manner. Twenty-four hr later, blood was taken and serum developed for 16 hr. Barbital buffer pH 8.6, mA = 7.5. The unknown compound appeared between α_1 and α_2 globulin. Trypan blue remained at the origin. ALB = albumin, α_1 = α_1 globulin, α_2 = α_2 globulin, U = unknown component, β = β-globulin, γ = γ-globulin, T.B. = trypan blue.

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TABLE I. Protein Fractions in Serum of Trypan Blue-Treated and Normal Animals. Determined by paper electrophoresis in barbital buffer, pH 8.6. Development was for 16 hours at 7.5 mA or 20 hours at 10 mA.

		%					
			Globulin				
Groups	No. of animals	Albumin	α-1	α-2	β	γ	Unknown compound
Normal controls	6	30.1 ± 1.4†	21.6 ± 1.2	8.7 ± .5	20.6 ± 1.4	12.4 ± .8	* 6.7 ± .2
1 hr‡	6	22.0 ± 2.9	22.8 ± 1.0	8.7 ± .4	20.2 ± 2.0	14.6 ± .6	11.7 ± .8
6 " ‡	5	22.6 ± .7	23.5 ± 1.2	8.2 ± 1.1	18.7 ± 2.5	15.8 ± 2.3	11.3 ± .2
24 " ‡	5	23.3 ± 1.9	18.7 ± 2.0	8.6 ± .9	23.7 ± 1.1	13.9 ± .5	11.8 ± .7
4 days§	6	24.8 ± .4	19.5 ± .8	8.2 ± .5	19.6 ± .7	11.6 ± .6	16.3 ± .7
6 " §	6	17.5 ± 1.1	25.0 ± .9	9.3 ± 1.1	21.7 ± 1.0	12.7 ± 1.0	13.9 ± .8

* Area which corresponded to location of abnormal protein and stained diffusely without showing any special peak was arbitrarily measured.

† Mean and stand. error.

‡ After single inj. of 4 ml of 0.5% trypan blue solution.

§ Repeated inj. of trypan blue, 4 ml every 12 hr.

even as short as 1 hour after the injection of the dye and remained fairly constant for 6 and 24 hours, but seemed to increase a little after repeated injection of the dye for 4 or 6 days (Table I). The amount of α-2 globulin, which migrated close to the abnormal component at pH 8.6, was quite constant in all of the experiments, while amount of albumin decreased markedly in all groups. It was difficult to detect any quantitative relationship between the abnormal component and proteins other than albumin, which might indicate a possible transformation of some protein(s) to the abnormal component.

Serum mixed with the same amount of trypan blue to be expected from the quantities injected *in vivo* was placed in a Dubnoff shaker for 5 hours at 39°C. No change was observed in the electrophoretic pattern, however. In another experiment serum and trypan blue were mixed and allowed to stand for 7 hours at room temperature. Again no change was produced in the electrophoretic pattern. It was also found that incubation of a serum-trypan blue mixture with liver slices did not produce this protein. Erythrocytes were hemolyzed in the presence of trypan blue and again no abnormal compound was formed.

Discussion. A correct interpretation of this phenomenon is not possible at this time. Three possible hypotheses are: (1) Trypan blue changes the electron charge of protein(s) and thus an abnormal electrophoretic pattern can be elicited. (2) Trypan blue stimulates synthesis of an abnormal protein or abnormal degradation of protein in some tissues of the body. (3) Trypan blue stimulates release of the "abnormal" protein stored in certain cells, resulting in a marked increase of its concentration in the blood.

In vitro experiments designed to discriminate among these 3 hypotheses indicated that the latter 2 appear more likely. It is not possible to say at what site this abnormal protein may be formed, nor whether this compound has any relation to thyroxine-binding globulin, but it is of interest that both are located between α-1 and α-2 globulin, and radioactive thyroxine is concentrated in this area whether the abnormal component is present or not(unpublished).

In relation to the appearance of this abnormal protein, it is of interest to recall the teratogenic effect of trypan blue(6). It might be possible that the abnormal protein metabolism produced by trypan blue has a causative role in producing abnormal fetuses, although the exact relationship between such abnormal protein metabolism and teratogenesis should be studied in pregnant animals treated with trypan blue before any conclusion can be reached.

Summary. Serum obtained from trypan blue-treated rats showed an abnormal protein band between α-1 and α-2 globulin on paper electrophoresis, which could best be separated at pH 9.2. The compound appeared within one hour and was present for several days. The quantity of this material in serum was greater than that of α-2 globulin as short as 1 hour after trypan blue injection and seemed to increase with repeated injections of trypan

blue. The compound was not formed *in vitro* by liver slices, erythrocytes or serum when incubated with trypan blue.

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Electrolyte Levels of Rat Salivary Secretions.* (25018)

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Recent work on rat salivary glands has revealed differences in levels of sodium and potassium in the 2 gland pairs (submaxillary-sublingual and parotid)(1). Resting submaxillary-sublingual has a high level of potassium and low sodium; resting parotid, on the other hand, exhibits a high sodium and low potassium. Stimulation of these glands over a 20 minute period, by pilocarpine, results in a loss of sodium by the parotid, while the submaxillary-sublingual loses potassium. In view of these differences in gland levels and pattern of behavior on stimulation, the electrolyte levels of submaxillary-sublingual and parotid salivas were investigated for possible correlation between electrolyte levels in the saliva and those of glands.

Methods. Adult male Long-Evans rats, weighing approximately 350 g, were fasted for 48 hours, and following light anesthetization with Nembutal, parotid or submaxillary-sublingual ducts were exteriorized, pilocarpine nitrate (2.2 mg) was subcutaneously administered, and the secretions collected from the freed ducts. To avoid contamination by residue in the ducts, the first drops were discarded. The duct was then led into a tared vessel covered with Parafilm. Following a timed collection period, usually 3 minutes, the vessel was reweighed, the secreting gland removed, and weights of both were recorded. Flow is thus expressed as mg/min or mg/min/mg of gland. An alternate collection method involved recovery of saliva from the oral cavity of animals from which one gland pair had previously been removed. Minor glands were found to contribute negligible quantities of fluid during short periods of stimulation. Electrolyte levels of each secretion were similar for the 2 collection methods during the short period of stimulation. Collection from the oral cavity was possible with restrained, unanesthetized animals; hence, this method was employed primarily to investigate possible effects of anesthetic on saliva electrolyte levels. Blood samples were removed from the jugular vein. After proper dilution of serum and salivary secretions, determinations of sodium and potassium were made by flame photometry and are expressed as milliequivalents per liter of secretion or serum.

Results. Representative values for electrolyte concentrations as well as flow rate during an initial 3 minutes of collection of submaxillary-sublingual and parotid gland secretions from the appropriate ducts are given in Table I. Sodium levels of submaxillary-sublingual secretions are low (generally less than 1/10 that of serum), and, while there may be as much as a 2-fold variation in Na levels among animals, it is apparent from Table I that such differences, in this time period, are not functions of gland size or secretion flow rate. Potassium levels of this secretion are very high, and again individual variation among animals is not correlated

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* This investigation supported by grant from Nat. Inst. Health.

TABLE I. Electrolyte Levels of Rat Salivary Secretions* and Serum.

	Na, meq/l	K, meq/l	Flow rate, mg/min./mg	Total Na + K, meq/l
Submaxillary sublingual	16.3	57.5	.018 (282)‡	74
	12.5	45.0	.056 (324)	58
	12.5	62.5	.075 (362)	75
	10.0	50.0	.013 (250)	60
	7.5	50.0	.052 (298)	58
Mean	11.8	53.0		65
Parotid	143	19.5	.075 (188)	163
	135	17.0	.047 (256)	152
	143	22.5	.110 (208)	166
	140	16.5	.048 (142)	157
	143	17.0	.079 (240)	160
	148	18.5	.029 (278)	167
Mean	142	18.5		161
Serum†	143	4.9		148

* Stimulation by pilocarpine of nembutalized animals; fluid collected for 3 min. from duct.
† Mean values (meq/l serum) for 4 animals.
‡ Numbers in parentheses are gland wt in mg.

with gland size or secretory rate of flow. Although potassium concentration of this secretion is approximately 10 times greater than that of serum, total Na + K concentration of saliva is appreciably less than for serum. Furthermore, even if the reported saliva calcium concentration is included in calculation of electrolyte content of this saliva(2), submaxillary-sublingual saliva is markedly hypo-osmotic to serum. Electrolyte pattern in the secretion of this gland complex generally resembles that of submaxillary secretion of dog (3) and submaxillary and parotid of man(4).

In parotid gland secretion, obtained also by duct collection during an initial 3 minute period of flow, sodium level is relatively high, closely approximating that of serum, and in contrast to flow rate, shows little variation among individual animals. Potassium concentration of parotid saliva is low compared with that of submaxillary-sublingual saliva but nonetheless represents at least a 3-fold increase over potassium level of serum and shows no correlation with gland size or, for this period, fluid flow rate. Total Na + K concentration in each case recorded (Table I) exceeds that of blood serum. Similar results have been obtained for parotid secretion collected from unanesthetized animals. Sodium-potassium pattern of parotid gland secretion of the rat thus bears a certain similarity to that of sheep parotid(5), pancreas(6), human lacrimal gland(7) and, possibly, rabbit parotid gland(8).

Summary. Rat submaxillary-sublingual and parotid gland secretions, collected directly from salivary ducts for initial period of 3 minutes during stimulation by subcutaneously administered pilocarpine, exhibit patterns of sodium-potassium concentration that differ markedly from each other. Submaxillary-sublingual saliva is essentially hypo-osmotic to serum, with sodium concentration of 12 meq/l and K of 53 meq/l, while parotid saliva has a total sodium-potassium concentration above that of serum (Na, 142 meq/l; K 18.5 meq/l). A broad correlation between gland sodium and potassium levels, together with changes on stimulation, and levels of these electrolytes in secretions from these glands is indicated.

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Polysaccharide Capsule of *Bacillus megaterium*.[*] (25019)

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By using immunochemically defined antibodies a complex capsule was discovered in a special strain ("M") of *Bacillus megaterium* (1-5). The bulk of the capsular material consisted of glutamyl polypeptide apparently enclosed in a polysaccharide "frame-work," the latter being chemically similar to the cell wall. Baumann-Grace and Tomcsik(7) studied 54 strains of *B. megaterium* belonging to 38 polysaccharide types but found a capsular structure similar to Bacillus "M" in only 10 of them. Polypeptide and polysaccharide components were, however, found in the capsules of all the strains although the location of the specific polysaccharide varied. Tomcsik (6) concluded that bacterial capsule usually differs chemically from the cell wall but that exceptions may occur as, for example in *B. megaterium*. More recently, however, Wilkinson(8) reviewing extracellular bacterial polysaccharides is not inclined to admit that *B. megaterium* is an exception and states rather categorically that "the cell wall and the predominant capsular components are quite distinct." Since Wilkinson did not reach this conclusion on the basis of any of his own experimental work, we wish to report on some new observations which show that under certain conditions strains of *B. megaterium* are able to produce a capsule consisting apparently entirely of polysaccharide serologically indistinguishable from the cell wall in which no trace of glutamyl polypeptide can be detected.

Results. All 54 strains of *B. megaterium* examined produced a complex glutamyl polypeptide-polysaccharide capsule 1-2 years after isolation when cultured on medium such as "G.F." Gladstone Fildes agar(7), which is rich in amino acids. Three to 4 years after isolation, however, most of the strains had lost this ability and even those which continued to produce polypeptide on GF agar failed to do so on Grelet's medium(9). The latter is a synthetic medium recommended for study of the sporulation of *B. megaterium* which contains glucose and nitrate as the sole sources of carbon and nitrogen. In this synthetic medium, although no polypeptide is formed, production of the polysaccharide part of the capsule is unimpaired and in 16 hr shaken cultures often reaches the dimension of that of Mg 45 shown in Fig. 1.

The results listed in Table I were obtained from cultures shaken for 16 hr in T-tubes(10) at 28°C after examination of the cells with both homologous polysaccharide and heterologous glutamyl polypeptide antibody. Older cultures were not usually suitable as sporulation had commenced and the capsules had usually been dissolved. The strains listed here were selected because, although no polypeptide was produced in the ordinary Grelet medium, addition of 0.5% asparagine was sufficient to stimulate its production. The "M" type of *B. megaterium* could not be examined as it failed to grow in Grelet's medium even in the presence of asparagine.

Discussion. Table I shows that same strains of *B. megaterium* produce under certain circumstances a capsule consisting entirely of a single component serologically identical with the cell wall polysaccharide. It is incompre-

TABLE I. Specific Capsular Reactions of 10 *B. megaterium* Strains Grown in Grelet's Synthetic Medium in Presence and Absence of Asparagine.

Medium:	With asparagine		Without asparagine	
Strain	Ps[*]	Pp[†]	Ps	Pp
Mg 6	+	+	+	—
" 7	+++	++	++	—
" 11	+++	+	+++	—
" 13	+	+++	+	—
" 16	+++	+++	+++	—
" 20	—	++	—	—
" 25	+	+++	+	—
" 26	—	+	—	—
" 29	++	+	+++	—
" 45	+++	+++	+++	—

[*] Ps = homologous polysaccharide type specific antibody.

[†] Pp = glutamyl polypeptide antibody.

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[*] This investigation was supported by research grant from Swiss Federal Labour Office, Bern.

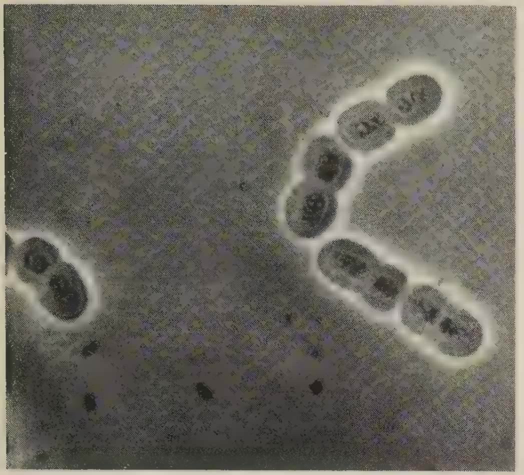

FIG. 1.

hensible that Wilkinson(8) based his assumption that the cell wall and capsular material are always chemically distinct, to some extent on the observations made by Jacox (11) as he postulated a non-specific salt-like linkage between excess cell-wall polysaccharide and polypeptide. Jacox(11) actually discovered a non-specific "swelling" reaction of the pneumococcal capsule which was only positive at pH 4.0 in presence of proteins and a NaCl concentration of less than 0.02 M. A similar reaction was not obtained with any other encapsulated organisms but only pH 4.0 was examined. Subsequently, however, Tomcsik and Guex-Holzer(12) described the pH dependent salt-like combination between bacterial capsular substances and various proteins.

The results here described also show that certain strains of *B. megaterium* do not produce glutamyl polypeptide in a synthetic medium if nitrate is the sole source of nitrogen and addition of asparagine is necessary for its production. This observation, however, requires confirmation with freshly isolated strains which produce capsules composed predominantly of polypeptide in media rich in amino acids.

Summary. Certain strains of *B. megaterium* produce capsules consisting of polysaccharide and lacking polypeptide if cultured in a synthetic medium containing glucose and nitrate as sole sources of carbon and nitrogen. The capsules give a specific capsular reaction with the same homologous antibody as that which reacts with the cell wall.

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Mode of Action of Trypsin on *Bacillus megaterium.** (25020)

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We described(1-3) our findings of the action of trypsin on cells of *B. megaterium.* Our results differed from those obtained by most other workers and suggested that the cytoplasm of the majority of strains of *B. megaterium* could be slowly but completely digested with trypsin at 37°C without previous heat treatment (curve 3). Several crude commercial preparations of trypsin were compared, but owing to the necessarily long in-

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* This work was aided by a grant from Swiss Federal Labour Office, Bern.

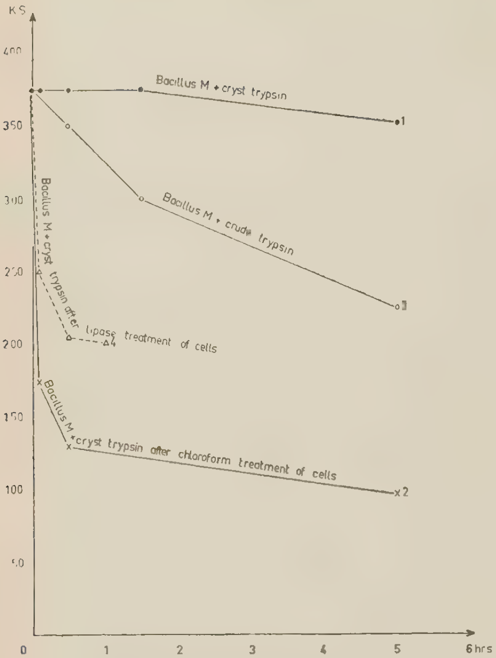

FIG. 1. Klett-Summerson readings (blue filter No. 42) of a suspension of a *B. megaterium* strain (Bacillus M), time calculated after addition of trypsin.

cubation time, chloroform was usually added as a preservative. The possibility that the chloroform played a role in the proteolytic digestion of the cells was not then suspected as control experiments with crude trypsin in which the chloroform was omitted yielded the same results. The small amount of pure crystalline trypsin which was then available to us (and for which we express our thanks to Mrs. E. Work, London) was examined only in the presence of chloroform. Since the publication of these results we have had the opportunity to study in more detail the action of crystalline trypsin (Mann) as well as of a purified preparation of the proteolytic enzyme elastase† on the cells of *B. megaterium* strain "M".

Methods. A moderately thick suspension of cells from a 16-hour culture of Bacillus *M* (Klett Summerson reading 375, with blue filter No. 42) in phosphate buffer M/30 pH 8.0 containing 1:16,000 dilution of either of these purified enzymes when incubated at 37°C showed no drop in turbidity even after 5 hours incubation. The addition of one drop of chloroform/ml of suspension, however, was sufficient to produce a marked drop in turbidity within 30 min. on further incubation and a microscopical examination showed an advanced digestion of the cytoplasm. Since the presence of chloroform interfered with the K.S. readings, the effect of pretreating the cells with chloroform before incubating with the enzymes was examined. One volume of cell suspension in buffer was shaken mechanically at room temperature with 1 vol. of chloroform. After removal of the chloroform layer and addition of enzyme to the cell suspension a rapid fall in turbidity (curve 2) and a marked digestion of the cytoplasm occurred within 5 min. and had almost reached a maximum in 30 min. Control cells treated with chloroform alone remained stable and showed no signs of cytoplasmic degeneration. Subsequent experiments showed that as little as one minute contact with chloroform was sufficient and that other organic solvents such as ether, butanol, 95% alcohol, acetone and toluol were equally effective. Chloroform could also be replaced, though less effectively, with 5% Tween.

Results. Reagents such as chloroform and butanol probably act directly on some lipid component of the cell surface, damaging it in such a way as to allow access of trypsin to the protoplast. The chloroform-treated cells were, in fact, found to be non-viable and on subsequent addition of lysozyme in buffered sucrose could not be transformed into spherical protoplasts but remained fixed in the rod form, suggesting that the cytoplasmic membrane had been damaged(4). Since, in contrast to crystalline trypsin, crude trypsin slowly digested the cytoplasm of untreated cells, it seemed probable that it was contaminated with a substance which acted in a similar way to chloroform. A spot test(5) for presence of lipase was weakly positive with the crude trypsin, so the combined action of lipase and crystalline trypsin was then examined.

The following 4 lipase preparations (Mann's Research Chemicals) were available: a relatively pure lipase from wheat germ, 2 crude pancreatic lipases and a crude lipase of microbiological origin. The wheat germ lipase was, unfortunately, found to be unusable as it contained a lysozyme-like enzyme which dissolved the isolated cell walls of *Bacillus M.* The results obtained with the pancreatic lipases were interesting, however, as it was shown that both lipases slowly digested the cytoplasm without previous or subsequent treatment of the suspension. Cells in phosphate buffer pH 6.6 containing 1:1,000 dilution of the lipase showed a gradual drop in turbidity of the suspension and microscopic examination after 5½ hours incubation at 37°C revealed almost complete digestion of the cytoplasm. Subsequent treatment of these lipase treated cells was, therefore, not possible but examination of the pancreatic lipases by the technic of Kolmer and Boerner (6) showed that both contained large quantities of trypsin. The lipase of microbiological origin, however, proved to be more suitable for experiments involving subsequent treatment of the cells with trypsin.

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† We would like to thank Dr. I. Balbó for kindly providing us with a sample of elastase and for cooperating in preliminary experiments.

Bacillus "M" was incubated in phosphate buffer at pH 6.0 containing 1:1,000 microbial lipase for 30′, at 37°C. No drop in turbidity or alteration in cell structure could be observed after this time and the cells were centrifuged down and resuspended in the same amount of phosphate buffer at pH 8.0 containing 1:10,000 crystalline trypsin. Within 5 min. there was a noticeable drop in turbidity which 60-120 min. had fallen by more than 40% (curve 4). Microscopically the cells showed a marked, although not complete digestion. A more prolonged incubation with trypsin or a longer treatment with lipase were not possible owing to instability of the controls.

Discussion. These results show that both crude lipase containing a high concentration of trypsin, as well as impure trypsin which probably contaminated with lipase can bring about digestion of the cytoplasm of untreated cells of *Bacillus "M"*. Pure crystalline trypsin alone, however, had no detectable effect, but preliminary treatment of the cells with an organic fat solvent such as chloroform rendered them immediately susceptible. In addition, a lipase of microbiological origin which was ineffective alone, behaved similarly to chloroform. It seems probable, therefore, that treatment of the cells of *Bacillus "M"* with lipase or with a fat solvent removes some lipoid material from the surface of the cell and allows subsequent access of trypsin to the protoplast. A further study of the problem would necessitate an examination with a purified preparation of lipase.

Summary. Our previous results, demonstrating with crude trypsin the digestibility of *B. megaterium* and the non-digestibility of *B. cereus,* could be confirmed with crystalline trypsin only by pretreating the bacteria with fat solvents or by adding lipase to it. The surface of *B. cereus* is different from that of *B. megaterium*; a lipid material can be removed from the surface of the later one which renders the bacterial protoplasts accessible to the action of proteolytic enzymes.

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Effect of B-Vitamins on Biosynthesis of Ascorbic Acid from Glucose Cycloacetoacetate in Germinating Phaseolus Radiatus.* (25021)

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The authors(1) have shown that glucose occurs in combination with acetoacetate in germinating mung beans and that thiamine and pantothenic acid accelerate such biosynthesis. Reed(2) reported that co-carboxylase and CoA which contain thiamine and pantothenic acid respectively, are involved in metabolism of keto-acids and may thus help condensation of glucose with acetoacetate to form glucose cycloacetoacetate. Sure *et al.*(3) showed that deficiency of thiamine results in reduced storage of ascorbic acid in various tissues and endocrines, and Kennaway and Daff(4) reported that there is less ascorbic acid in the livers of mice kept on thiamine deficient diet. Smita *et al.*(5) have also observed that thiamine stimulates ascorbic acid biosynthesis in germinating mung beans and Thangamani and Sarma(6) showed that thiamine and pantothenic acid are also involved in greater biosynthesis of ascorbic acid from glucose cycloacetoacetate in germinating mung beans than with glucose alone. That reducing substance found during the process of germination of mung beans is ascorbic acid has recently been confirmed by the authors (7) through paper chromatographic technic.

Methods and Materials. Germination of mung beans was carried out as reported(1). Acetoacetate was added in the form of its sodium salt and in equimolar proportion with glucose. Glucose cycloacetoacetate (GCA) was prepared according to the method of West(8) as modified by Nath *et al.*(9). Due to its insolubility in cold water, it was dissolved in hot water and its solubility remained to the extent of 100 mg per 10 cc in cold condition. Pantothenic acid was used as Ca salt. After germination, the selected seeds were harvested and macerated with metaphosphoric-acetic acid mixture and their ascorbic acid contents estimated by the indophenol dye method(10). The results obtained after germination of the mung beans for different periods in presence of glucose and acetoacetate and glucose cycloacetoacetate with and without vitamins and their anti-vitamins are shown in Tables I and II.

Results. Both pyrithiamine and pantoyltaurine inhibit ascorbic acid synthesis in presence of acetoacetate and glucose (Table I), the inhibition being checked by the corresponding vitamins. Results with glucose cycloacetoacetate are represented in Table II.

Discussion. The role of thiamine and pantothenic acids as co-factors in bringing about

TABLE I. Effects of Pyrithiamine and Pantoyltaurine and Corresponding Vitamins on Biosynthesis of Ascorbic Acid from Glucose and Acetoacetate during Germination of Mung Beans.

	Fresh basis					
	mg/100 seedlings after			mg/100 g after		
Substances in germinating medium	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
Nil	1.15	2.05	2.25	9.2	10.25	11.25
Acetoacetate + glucose	1.54	3.82	4.14	15.4	24.83	24.84
Idem + pyrithiamine	1.4	3.3	4.01	14.0	21.4	24.06
" + " + thiamine	1.51	3.85	4.33	15.48	24.52	25.98
" + pantoyltaurine	1.3	3.11	3.65	13.0	20.21	21.9
" + " + pantothenate	1.85	3.97	4.69	18.5	25.8	28.14
" + thiamine + pantothenate	2.13	4.27	5.16	21.3	27.7	30.96

Concentrations: Acetoacetate (sodium salt), 70 mg/10 cc medium; glucose, 100 mg/10 cc; and 200 PPM each of pyrithiamine, pantoyltaurine, thiamine and calcium pantothenate.

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* The authors record their grateful thanks to the Council of Scientific & Industrial Research for a Research Grant. Their thanks are also due to Mr. R. G. Joglekar of the Agricultural Department, Nagpur for supply of good quality phaseolus radiatus.

TABLE II. Effects of Pyrithiamine and Pantoyltaurine and Corresponding Vitamins on Biosynthesis of Ascorbic Acid from Glucose Cycloacetoacetate (GCA) during Germination of Mung Beans.

	Fresh basis					
	mg/100 seedlings after			mg/100 g after		
Substances in germinating medium	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
Nil	1.85	1.92	2.2	6.8	10.36	11.2
Glucose cycloacetoacetate (GCA)	1.46	2.8	2.1	11.48	16.8	24.6
Idem + pyrithiamine	1.36	2.23	3.51	10.88	13.38	21.06
" + " + thiamine	1.45	2.82	4.2	11.6	16.92	25.2
" + thiamine	1.53	3.25	4.4	12.24	26.0	27.65
" + pantoyltaurine	1.3	2.4	3.9	10.4	14.4	23.5
" + " + pantothenate	1.65	2.86	4.4	13.2	17.16	26.4
" + pantothenate	1.8	3.25	4.5	14.4	26.0	28.7

Concentrations: Glucose cycloacetoacetate, 100 mg/10 cc medium and 200 PPM each of pantoyltaurine, pyrithiamine, thiamine and calcium pantothenate.

enzymic biosynthesis of ascorbic acid from glucose cycloacetoacetate is indicated. These vitamins may be taking part both in condensation of glucose with acetoacetate to form GCA and in the further reaction leading to biosynthesis of ascorbic acid. In absence of these vitamins, the condensation reaction is hampered resulting in a low value of combined sugar, as reported previously, and consequently in lower output of ascorbic acid.

Summary. The anti-vitamins, *i.e.* pantoyltaurine and pyrithiamine, inhibited ascorbic acid biosynthesis from acetoacetate and glucose in germinating mung beans indicating a blocking of the condensation reaction of acetoacetate with glucose to form GCA. The corresponding vitamins reversed the inhibition by antivitamins. That pyrithiamine and pantoyltaurine inhibited synthesis of ascorbic acid in presence of GCA, and the corresponding vitamins reversed the inhibition. Cocarboxylase and CoA, of which thiamine and pantothenic acids are the constituents, are involved in both the condensation reaction of glucose with acetoacetate to form GCA and in the further metabolism of GCA to ascorbic acid.

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Ultrastructure and Site of Formation of Rabbit Papilloma Virus. (25022)

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Since its discovery in 1933(1), numerous attempts have been made to characterize physically the rabbit papilloma virus. The early conclusions from sedimentation, diffusion and viscosity measurements of purified virus protein preparation indicated an aspherical particle with a molecular weight of 47,100,000(2).

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In later studies with the electron microscope, where shadowed dispersions were examined, the virus particle was found to be essentially spherical with a diameter of 40 to 45 mμ(3,4). By using fluorescent labelled antibodies, Noyes and Mellors(5,6) have demonstrated that the virus is found only in the more keratinized cells of the epidermis. We now report electron microscopic studies of thin sections of tumor tissue and of purified ultracentrifuged pellets.

Materials and methods. Warts from Kansas cottontail rabbits which had been stored in glycerine-saline at 0 to 5° for 1 to 6 years, as well as warts experimentally induced in cottontail and domestic rabbits, were studied. With a stereoscopic microscope blocks were dissected and oriented so that thin sections could be cut which contained the dermo-epidermal junction and extended well up into the tumor. In this manner electron microscopical localization of the virus was possible. *Purification procedure.* Five g of glycerinated warts from which the dermis had been stripped were quite finely minced by chopping with razor blade on glass plate. The fragments, suspended in buffered saline (pH 7.2), were finely ground in Potter-Elvehjem homogenizer. The thick grey brei was then subjected to repeated cycles of fluorocarbon emulsification and low speed sedimentation using VirTis homogenizer (45,000 rpm) and a refrigerated centrifuge (300 g)(7,8,9). Cycles were repeated until greyish discoloration was no longer present in sedimented emulsion. At this point the faintly opalescent aqueous supernatant was centrifuged at 40,000 rpm (100,000 g) for 1 hour to yield a pellet. The extreme bottom of pellet was dark, indicating presence of some melanin granules. The major part of pellet was gelatinous consisting of small freon micells. A white coat, thicker at upper side of the pellet, overlaid the gelatinous layer. Fixation with 1% buffered OsO_4 caused the white coat to turn brown. The upper thick portion of this layer was carefully isolated, dehydrated with alcohol and embedded in methacrylate. Thin sections were examined in the electron microscope.

Results. Both the embedded tissue and embedded pellets revealed profiles of a relatively uniform class of particle having diameters of 25 to 35 mμ. The particles in the 2 types of preparation were entirely similar. Sections containing the particles are illustrated in Figs. 1 to 3.

Location of virus. Virus was found sporadically in cells from a short distance above basal layer to the surface, although it was more difficult to identify in most cornified cells. Determination of the original exact intra-cellular location was impossible because of lack of preservation of fine structure of glycerinated tissue. Viral particles were present as a pool lying in the central portion of cells among fine keratin fibers and melanin granules (Fig. 1). Cell boundaries were thick and heavily keratinized.

Structure of virus particles. The 4 different profiles in Fig. 2 may possibly result from

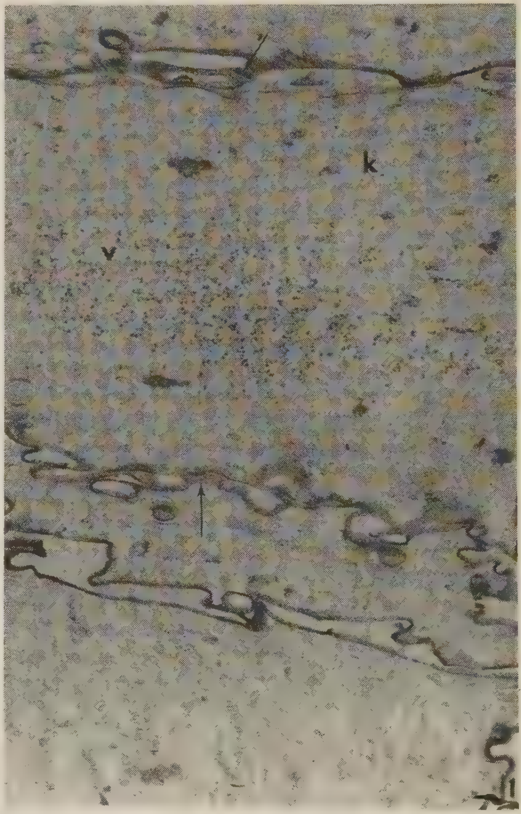
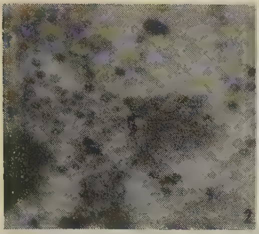
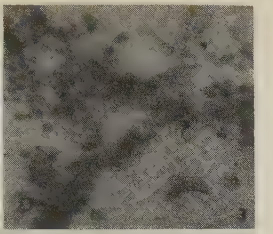
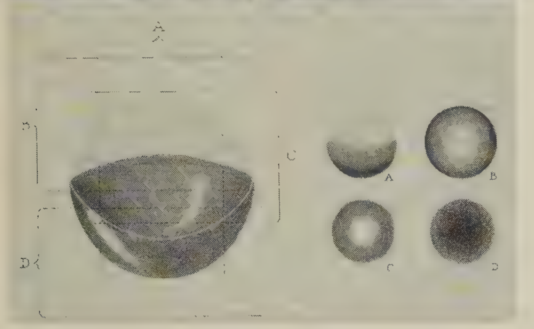


FIG. 1. Survey field showing a virus pool, v, surrounded by keratin fibers, k. Note apparent lack of infection in some cells as contrasted with large numbers of particles in others. Two of the many cell contact areas (desmosomes) are indicated by arrows. × 18,600.







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Electronmicrographs have inadvertently been reduced in size so that the fine structure has been lost. *(The editors)*

FIG. 2. Very thin section of an osmium fixed purified pellet showing variation in profiles of the virus. See Fig. 4 for explanation. × 45,000.

FIG. 3. Same as Fig. 2 except a thicker section. Particles appear to have uniform density. ×45,000.

FIG. 4. Schematic diagram illustrating the possible effect of plane of section on appearance of assumed non-homogenous viral bodies. To give the profile represented in A, the plane of section is vertical in the large sphere and the beam passes horizontally. To give profiles B, C, and D, the sphere is sectioned horizontally at levels indicated.

different aspects of the same particle as illustrated in Fig. 4. A particle's appearance is affected by its vertical position in the section, the extent being dependent on thickness of the section. It is difficult to estimate thickness of sections by interference colors when they are under 50 mμ, because sections thinner than this all appear grey when viewed with white light on a liquid surface(10). In studying serial sections of vaccinia virus(11), one virus particle (235 mμ diameter) required 7 or 8 sections to traverse it. This indicated an average section thickness of 30 to 33 mμ. It is likely that the section represented in Fig. 2 has a thickness less than the diameter of the particles, whereas that of Fig. 3 is probably greater. The particles of Fig. 2 show more knife distortion, which is characteristic of very thin sections, than do those of Fig. 3. The difference in appearance of the various particles of Fig. 2 can be explained by assuming a non-homogenous density in the spheres as illustrated in Fig. 4. It is therefore likely that all the profiles illustrated are different aspects of very similar particles. In the thicker section of Fig. 3 most particles appear to have a uniform density because more of them lie wholly within the section.

Discussion. The profile diameter most frequently observed in our sections is about 33 mμ. This measurement is significantly less than those reported in the literature where measurements were made on air dried or frozen dried dispersions. Electron microscopy does not permit a true measurement of biological substances in their natural state and it is likely that most of the discrepancy results from the methods of preparation. The same kind of discrepancy occurred with other viruses. Influenza virus was about 100 mμ(12) in dispersion and 65 mμ in fixed sections(13). Similarly the mouse mammary carcinoma agent was about 130 mμ to 150 mμ in unsectioned, air dried preparations(14), whereas in embedded and sectioned preparations its average size was only 100 mμ(15). The unsectioned bodies may appear large because of flattening and because of a coat of protein or other material adhering to them. On the other hand, alcohol dehydration and methacrylate embedment cause appreciable shrinkage of sectioned specimens.

In attempting to locate the site of viral development a rather thorough search was made of tissues fixed immediately after removal from both wild and domestic rabbits. So far, however, we have been able to identify the viral bodies only in tissues that were soaked several months in glycerine. In the freshly fixed papillomas granules and particles were so abundant that identification of the virus was difficult and uncertain. Soaking in glycerine dissolved away enough cellular material to leave the virus distinctly visible. It has been well established that storage in 50% glycerine preserves viral activity even for several years, but a great deal of cellular contents appears to be lost, leaving the virus readily visible. All of our preparations have been tested and found to be biologically active. A quantitative correlation of the activity with particles and the effect of preparative procedure on activity in both wild and domestic rabbits will be reported elsewhere.

Summary. Thin sections of rabbit papillomas reveal pools of dense virus particles in only highly keratinized cells. Viral bodies were readily distinguishable only in papillomas which had been soaked in glycerine-saline for some time. Viral bodies in fresh warts were masked by keratin, melanin and other granules. The bodies are spherical with a non-homogeneous density. They appeared to vary from 25 to 35 mμ in diameter, the most frequent size being 33 mμ. Particles in tissues soaked 1 year or 6 years were identical and equally active in test animals. Sectioned pellets of virus purified by a fluorocarbon process revealed the same particles as those seen in the tissue.

Since submitting this paper we have found the virus in fresh papillomas from cottontail rabbits. It is first seen in the nucleolus, but later fills the entire nucleus and may form crystalline-like arrays.

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A New Virus of Ducks Interfering with Development of Malaria Parasite (*Plasmodium lophurae*). (25023)

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The avian malaria parasite *Plasmodium lophurae* has been maintained for 14 years in my laboratory by weekly transfer in young ducklings of infected blood. In Feb. 1958 my technician, Mary Chichester, and I noted that in one passage the infections lost their synchronicity(1) and failed to develop with usual severity, and the parasites appeared abnormal. On a subsequent passage parasitemias remained very low but the ducklings died. Since these deaths could not have been a result of malarial infections it appeared likely that some other infective agent had been present in a duckling used for passage of malaria and that this agent had been enhanced in virulence by rapid passage. The following experiments were therefore undertaken to separate this agent from the malaria parasites, to characterize the agent, and to study the effect of introducing the agent into ducks simultaneously infected with *P. lophurae*.

Experimental. 1. *Recovery of P. lophurae free from contaminating agent.* Blood was taken from a duckling showing 17 parasites, many of them abnormal, per 100 red blood cells, and mixed with one-tenth its volume of physiological saline containing heparin at 27 mg/100 ml. When the heparinized blood was inoculated to other ducklings these died in 5 days with parasitemia of 1/100 red cells. When blood cells were separated from plasma by centrifugation, washed with centrifugation 3 times in 0.85% sodium chloride solution, and finally resuspended in saline to original volume of the blood, inoculation of resulting suspension to a duckling produced 5 days later a parasitemia of 28/100 red cells, with only some of the parasites abnormal. Erythrocytes of this duck were washed in a similar way and inoculated to another which, 4 days later, had 17 parasites/100 red cells, most of them quite normal. Two more transfers in this fashion at intervals of 4 and 3 days respectively completed the process of diluting away the contaminating agent. Thereafter *P. lophurae* was again passed in series by weekly inoculation of infected whole blood appropriately diluted in saline. The parasites appeared normal and developed synchronously during the first 5 days of infection, just as they had done before contamination. The success of this procedure for freeing the parasites of contamination shows that contaminant was not present in either erythrocytes or parasites.

2. *Characteristics of infective agent.* The agent was easily obtained free from malaria parasites by subinoculation of plasma of a duckling infected with both entities. It was soon found that the infection could be transmitted readily by intravenous inoculation of plasma from an infected bird. White Pekin ducks, used throughout the work, were susceptible at all ages tested (3 days to 3½ months). Death generally occurred suddenly within 3 to 5 days after inoculation of as little as 0.1 ml of a 1:10 dilution of infective plasma into a 200 g duckling. With smaller doses which did not kill so quickly, the only signs of illness were weakness, loss of appetite and brown discoloration of bill. A few birds inoculated with very small doses survived the infection and were later immune to a dose of infective plasma which killed previously uninfected controls. For example, one duckling was inoculated with a 1:100 dilution of infective plasma. Eight days later this duckling was thin and had a brown beak, but after another 2 weeks it had recovered. This bird then showed no effect when it was challenged a month after initial inoculation with a dose of infective plasma which killed controls in 5 days. The infection in ducks was accompanied by severe anemia. Thus, two 3-month-old ducks which appeared weak 5 days after inoculation had a red cell volume of only 20% instead of the normal 35%. In younger ducklings anemia was generally even greater. Plasma of high infectivity had a greenish tinge and a characteristic, almost milky, cloudiness when viewed by reflected light. No consistent changes in leukocytes were noted.

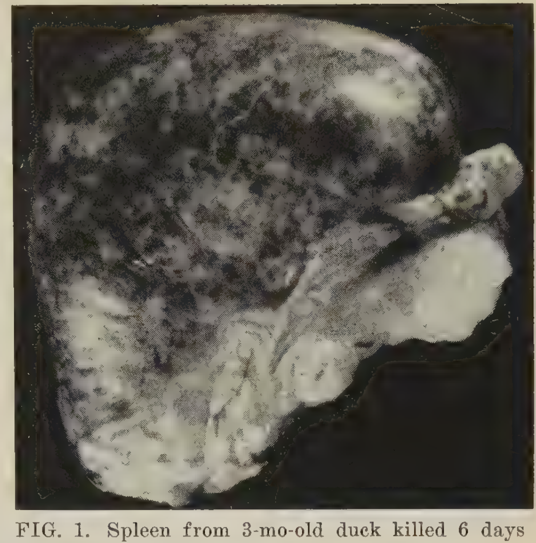

FIG. 1. Spleen from 3-mo-old duck killed 6 days after inoculation with spleen necrosis virus.

At autopsy a constant finding was enlarged spleen more or less full of pale whitish areas (Fig. 1). The liver was often enlarged and reddish, and sometimes showed hemorrhagic areas. Some deaths, but by no means all, were associated with obvious hemorrhage into the peritoneal cavity.

The disease was not contagious. No instance of infection of ducklings by contact occurred although there was ample opportunity for this.

Of four 2-day-old chicks inoculated with duck plasma which killed ducklings in 4 days, one died on 10th day. It had an enlarged liver and a white tumor-like lesion at one side of spleen. Another died on 19th day with no characteristic pathology. The other 2 survived and showed no lesions when killed a month after inoculation. The agent does not seem to infect chicks readily.

Plasma from infected ducks, stored in deep freeze several weeks or in dry-ice box for nearly a year, was still highly infective. Filtration of infective plasma through Selas 03 porcelain filter, known to retain bacteria, did not reduce its infectivity. In view of this fact

TABLE I. Neutralization Tests of Spleen Necrosis Virus.

Infective plasma diluted 1:5 in nutrient broth and incubated ½ hr at 37° with equal vol of:	Days to death*	
	Exp. A	Exp. B
Nutrient broth	13, 11	4, 4
Normal duck serum	12, 11	3, 4
Serum from recovered, immune duck	18, 26	9, 14
Anti-duck hepatitis serum	16, 14	4, 4

* In each experiment, each mixture was inoculated intrav. to two 5-day-old ducklings, 0.1 ml per duckling.

and of failure to obtain any cultivable organism from infective plasma, or to see any micro-organism by light microscopy, the infective agent is almost certainly a filterable virus.

3. *Neutralization of virus by serum from immune duck.* Two experiments (Table I) showed clearly that the virus was neutralized by serum from a duck which had recovered from initial inoculation and had proved immune to challenge with a dose fatal to control birds. Plasma of exceptionally low activity was used for Exp. A and of usual high activity for Exp. B. The virus was not neutralized by normal duck serum or by an anti-duck hepatitis serum obtained through the courtesy of Dr. E. Dougherty, 3rd, of Duck Disease Research Laboratory, Eastport, L. I., N. Y.

4. *Microscopic pathology.* Only the spleen and liver of ducklings near death from the viral infection have been studied. Tissues were fixed in Zenker with 5% formaldehyde and sections stained with hematoxylin-eosin. In the spleen there were obvious large necrotic areas corresponding to the whitish lesions visible grossly. One spleen had a region of still normal basophilic cells with numerous mitotic figures. The spleens were hyperemic as were also livers of diseased birds. In liver as in spleen there was necrosis. In addition, liver sections showed conspicuous cuffing of blood vessels with fairly numerous mitotic figures among these cells.

5. *Effects of virus on Plasmodium lophurae.* In ducklings in which a contaminating infective agent was first suspected, development of malaria parasites was sharply inhibited and many of the parasites were clearly abnormal. Abnormal parasites in blood films stained with Giemsa showed any or all of the following characters: diffuse or fragmented nuclear material; excessive, large, colorless vacuoles; generally pale or diffuse purplish stain; fragmentation of cytoplasm. Sometimes the parasite was completely broken down with only some pigment grains left within the erythrocyte. Some difficulty was encountered in trying to reproduce these effects by simultaneous inoculation of ducklings with the 2 separated agents—*P. lophurae* and the virus. With too large a dose of virus the ducklings would die in 3 days before any effects on malaria parasites were apparent. With too small a dose the malarial infection would reach a peak before striking effects of the virus could be seen. In several experiments, however, appropriate dosage was achieved, resulting in some reduction in numbers of malaria parasites, in appearance of abnormal parasites and in a disturbance of synchronous development. For example, in Exp. C (Table II) the control ducklings on 4th day had the usual highly synchronous infection with nearly all parasites uninucleate trophozoites. In presence of virus the parasitemias were somewhat lower and numerous abnormal parasites were present, but more important, moderate to large numbers of parasites were in the multinucleate stage, which had been passed by controls during the previous night. Similar retardation is shown in Exp. D, in which there was no effect on total parasite count. On 5th day, most parasites in control ducks were large multinucleate forms whereas in ducks which had received the larger dose of virus (No. 15-18) over half of the parasites were still young forms much as they had been on the previous day. A small retarding effect and fewer abnormal parasites were seen in the ducks which received the smaller dose of virus. In both experiments treatment with a mixture of folic acid, pantothenate and coenzyme A appeared partially to overcome the inhibitory effects of the virus, suggesting that the virus was in one way or another interfering with the nutrition of malaria parasites.

Discussion. The new transmissible disease of ducks here described may be designated spleen necrosis of ducks. It is characterized especially by its effect on the spleen, which becomes greatly enlarged and more or less

TABLE II. Retardation in Development of *P. lophurae* in 22 Ducks Simultaneously Infected with Spleen Necrosis Virus.

Exp.*	Virus, ml†	Parasites per 100 red cells	% parasites Uni-nucleate	% parasites Abnormal (all stages)
C	0	240	95	0
	0	540	90	0
	.05	87	61	23
	.05	140	19	35
	‡ .05	290	67	2
	.05	90	14	21
D	0	770	25	0
	0	110	7	0
	0	730	28	0
	0	425	26	0
	.025	610	26	4
	.025	540	31	6
	.025	660	30	2
	.025	480	28	10
	.05	700	50	4
	.05	570	69	7
	.05	540	85	13
	.05	650	54	11
	‡ .05	450	45	6
	.05	540	44	7
	.05	640	49	7
	.05	710	45	3

* Parasite counts are given, in Exp. C for 4th day after inoculation, and in Exp. D for 5th day.

† Virus inoculum was a 1:10 dilution in broth of infective duck plasma, given at indicated dosage/100 g body wt.

‡ Ducks 5 and 6 and 19-22 each received intrav. on each of days 1-4 after inoculation 1 ml of a solution containing, in mg/100 ml, folic acid 1.7, calcium pantothenate 70, liver coenzyme A concentrate 30 (15 units CoA/mg).

replaced by pale necrotic tissue. No disease of ducks with similar pathology has been previously reported. Duck viral hepatitis(2) has no marked effect on the spleen, although the enlarged liver with hemorrhagic areas characteristic of hepatitis is also often seen in spleen necrosis. The 2 entities may be further distinguished by failure of duck anti-hepatitis serum to neutralize the spleen necrosis virus. Infectious serositis of ducks, possibly caused by psittacosis-like organism(3), produces an enlarged, pale and mottled spleen, but there is also marked fibrinous inflammation of serous membranes and yellowish exudate over the liver, changes not seen in the new disease. Moreover, infectious serositis, like fowl cholera and duck plague, is highly contagious, whereas the spleen necrosis appeared not at all contagious.

The origin of the infection and its mode of transmission in nature are unknown. One might suppose that it is present in some ducks as a latent, egg-transmitted virus and that pathogenicity appeared as a result of rapid transfer at weekly intervals in connection with maintenance of strain of *P. lophurae*.

Effects of viral infections on the course of experimental malaria have been noted occasionally before. Jacobs(4) found that ducklings infected with ornithosis virus and inoculated 10-12 days later with *P. lophurae* mostly survived the latter infection. He attributed the effect to hyperplasia of the reticulo-endothelial system brought about by previous viral infection. It will be noted that the time relations here were quite different from those obtaining in the present study where both virus and parasites were inoculated simultaneously. Huff (personal communication) observed that malaria parasites in canaries infected with fowl pox became vacuolated and did not develop normally. In a more extensive series of observations, Yoeli *et al.*(5) found that West Nile virus interfered with development of *P. berghei* in mice. The parasites showed abnormalities like those of *P. lophurae* in ducks with spleen necrosis.

In no case has the mechanism of these effects been worked out. It may be that the virus prevents the parasite from getting some essential nutrient. Ability of a folic acid-coenzyme A supplement to counteract somewhat the effect of spleen necrosis virus on *P. lophurae* would be in line with such a hypothesis.

Summary. In the course of passage of *P. lophurae* in ducklings by weekly transfer of infected blood, a contaminating infective agent appeared which inhibited malarial infection and caused rapid death of ducks. The agent was present in plasma of infected birds and was readily separated from malaria parasites. It was a filterable virus, producing, in ducks inoculated with appropriate doses, a rapidly fatal disease characterized especially by enlargement and necrosis of the spleen. This spleen necrosis virus was neutralized by serum from a recovered immune duck but not by antiserum to duck hepatitis virus. In ducklings simultaneously inoculated with *P. lophurae* and a suitable dose of spleen necrosis

virus, development of malaria parasites was retarded and many of them appeared abnormal.

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Effect of Gastric Emptying Upon Propulsive Motility of Small Intestine of Rats. (25024)

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The influence of antral peristaltic activity upon motility of small intestine has been considered by Alvarez(1) and others before him. Existence of a feeding response alerting the entire intestine has been observed repeatedly in dogs with exteriorized loops(2), but this does not necessarily involve gastric motility for it has been observed in the sham fed(3). Obviously gastric motility is necessary for introduction of material before the intestine can propel it. However, we wished to determine whether the continued propulsion of the material is influenced by the activity of the stomach in emptying.

Material and methods. We further modified Macht's technic so that amount of intubated mixture in both stomach and intestine might be quantitated at end of experimental period. The mixture consisted of 1 g methylcellulose, 200 mg trypan blue, 20 g powdered tetrafluorethylene resin (Teflon) and 200 ml H_2O. The trypan blue was used as a marker, while Teflon was chosen because it is practically indestructible. Up to 3 ml of this mixture was given by gastric intubation in 38 unanesthetized male adult albino (Wistar) rats weighing 210 to 410 g and fasted overnight. After killing the rats at end of 20 min and determining the distance traversed by the blue mixture, the stomach and small intestine were digested separately in nitrosyl sulfuric acid upon a steam bath. The Teflon remaining was collected upon sintered gooch crucibles, and after washing with chloroform to remove traces of fat, was dried to constant weight. In 12 similar rats killed immediately after intubation, it was found that 10% of the Teflon had left the stomach at the moment of intubation and traversed 6% of the small intestine.

Results. From the amount of Teflon recovered from the intestine and total amount intubated (Teflon recovered from stomach plus that from intestine) the per cent gastric emptying was calculated. When this value for each rat was plotted against per cent of small intestine traversed the scatter diagram of Fig. 1 resulted. (For conservation of space, 6 extreme points to the left and above have been omitted from the Figure but not from the calculations.) Also plotted on the

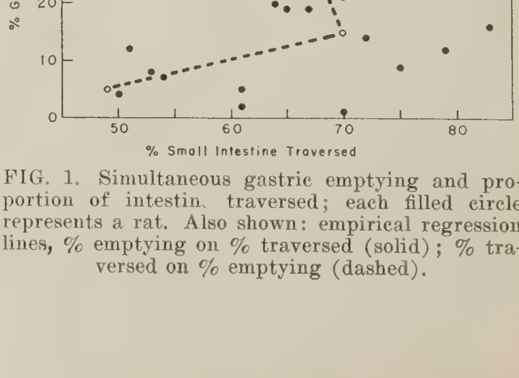

FIG. 1. Simultaneous gastric emptying and proportion of intestin. traversed; each filled circle represents a rat. Also shown: empirical regression lines, % emptying on % traversed (solid); % traversed on % emptying (dashed).

diagram are the empirical regression lines. The correlation coefficient is 0.003.

Conclusion. The extremely low correlation found in these experiments suggests that the propulsive motility of small intestine is essentially independent of that of the stomach.

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Precise Zone of Localization of Anti-Kidney Antibody in Various Organs.* (25025)

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Antibodies prepared against rat kidney, when injected intravenously into rats, are fixed by kidney and several other normal tissues, as shown by radiolabelled antibody technics(1,2). The precise structures on which localization takes place have been visualized by immunohistochemical means in kidney and adrenal(3,4) but not in other organs. Presence of antigens in various tissues, indistinguishable from kidney antigens has been shown. Radiolabelled antibody technics show that antisera against various tissues contained kidney localizing antibodies(1,2); these tissues are capable of absorbing out the localizing activity(5). Baxter and Goodman have shown that antisera to various tissues can produce glomerular lesions and that these tissues can neutralize kidney localizing antibodies(6,7). Cruickshank and Hill(8) demonstrated by immunohistochemical methods the presence of identical antigens in these tissues which react *in vitro* with antibodies formed against rat kidney when cells are sectioned and the antibody brought in contact with cell contents. The fact that cross reacting antigens are found in various organs does not mean that circulating antibodies have access to them. The present work was done to determine just which structures the *in vivo* administered antibody do give a high enough local concentration to be seen by immunohistochemical procedures.

Materials and methods. Rabbit anti-rat kidney serum was that used previously(9). A total of 4 female and 4 male rats weighing 150-200 g were injected intravenously in the tail with 2 ml of rabbit anti-rat kidney serum or normal rabbit serum (heat inactivated for 30 min at 56°C). All animals were sacrificed and perfused 18 hr after intravenous injection of antibody. Uninjected control rats were also used. Fluorescein and tetramethylrhodamine labelled horse anti-rabbit globulin were prepared as previously described(10) by procedures similar to those described by Coons and Kaplan(11). The preparations were absorbed 3 times with rat liver sediment to remove components which stained rat tissues nonspecifically(12). Immunohistochemical staining was performed as follows: Tissues were excised, frozen with solid CO_2 and stored at –20°C. Frozen sections 5 μ thick were cut and thawed on gelatinized slides; dried under a lamp and used on day of preparation. Sections were immersed in saline and the area around the tissue dried. A few drops of fluorescein or tetramethylrhodamine labelled horse anti-rabbit globulin were placed on the tissue and incubated at room temperature 1 hour. The sections were washed 10 min in buffered saline (pH 7) and mounted in glycerol containing 10% buffer. Fluorescence was observed through Reichert fluorescence microscopy equipment. When sections of uninjected rats were stained *in vitro* with rabbit anti-rat kidney antibody prior to treatment with labelled horse anti-rabbit serum, the rabbit antiserum was absorbed with human

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* This work was supported in part by U.S.P.H.S. through grant from Nat. Heart Inst.

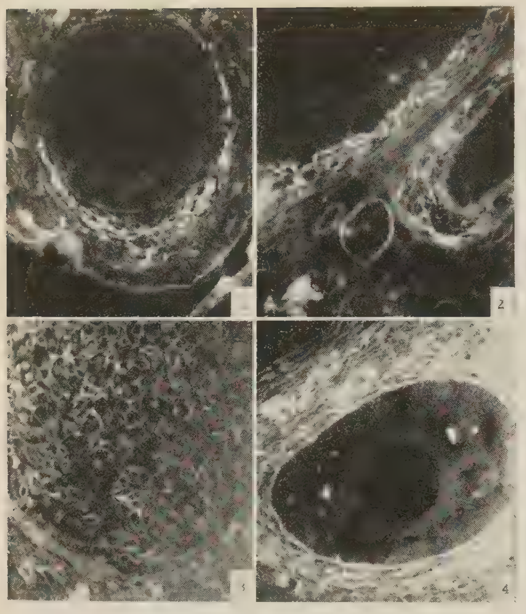


FIG. 1-3. Ovary from a rat inj. intrav. with rabbit anti-kidney serum. Presence of the localized antibody was developed with fluorescein labelled horse antibody to rabbit globulin. Fig. 1 shows staining around follicles and in vascular elements; follicular fluid and follicular cells are unstained. Fig. 2 shows part of a large follicle. Strong fluorescence is seen particularly in inner boundary of follicle. Follicular epithelium is unstained. Vessels and stromal elements are stained, the former somewhat more intensely. Fig. 3 shows staining of an atretic follicle, cellular elements within follicle are unstained but connective tissue elements appeared to be staining strongly indicating that *in vivo* the antibodies have ready access to these areas.

FIG. 4. Ovary section (from normal rat) treated *in vitro* with anti-rat kidney serum and staining developed as before. Major areas of staining are somewhat similar to that seen above. However, all the connective-tissue areas had a greater tendency to stain than is seen in the previous sections indicating that *in vivo* the antibodies did not have as ready access to these areas.

liver sediment to remove nonspecifically staining material. Normal rabbit serum so treated does not stain rat tissues nonspecifically.

Results. When rats were injected with anti-RK serum and assayed for fixed rabbit globulin by immunohistochemical methods, *kidney, adrenal, ovary* and *spleen* showed structures on which localization took place to a high concentration, *thyroid, lymph node* and *liver* showed localizations but of lower concentrations, while *testes, lung, skin, brain* and *heart* showed no staining. When sections of these organs of uninjected animals were stained *in vitro* by direct application of anti-RK serum, staining was essentially that described by Cruickshank and Hill(8). Those organs showing staining following injection of anti-RK serum are described below; figures are shown side by side of *in vivo* and *in vitro* staining.

Kidney and adrenal. In vivo localization of anti-kidney antibody in these organs has been discussed(3,4). Localization was limited to vascular structures and did not involve the multitude of other sites stained when anti-RK was placed directly on kidney sections, as described by Cruickshank and Hill(8). In this latter case, of course antibody is brought

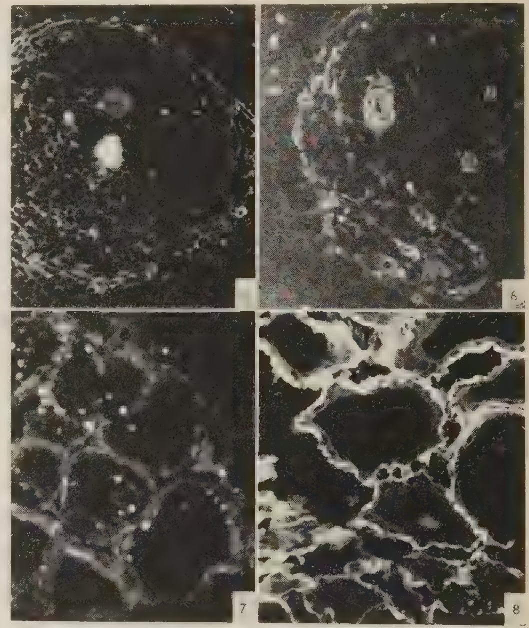
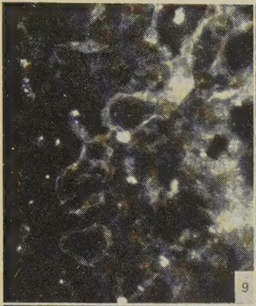


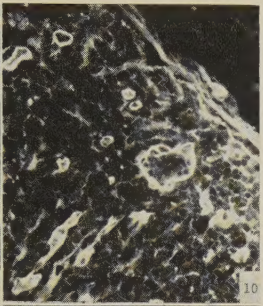
FIG. 5. Spleen from a rat inj. intrav. with anti-rat kidney serum. Fixation of antibody is seen in areas bordering the white pulp. Pulp artery seen in the field is unstained. Bright area directly below pulp artery is an artifact.

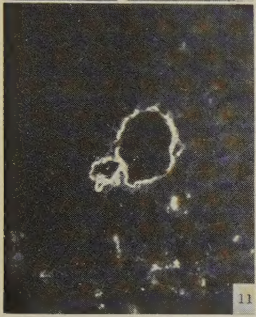
FIG. 6. Spleen section treated *in vitro* shows essentially the same type of staining as that described above, however, stroma supporting pulp artery is stained while smooth muscle coat is not.

FIG. 7. Thyroid from a rat inj. intrav. with anti-rat kidney serum. Weak staining is seen just below follicular epithelium while epithelium is unstained. Colloid was washed out in this section, however, when it was retained it remained unstained.

FIG. 8. Thyroid section treated *in vitro*. A similar strong staining is seen in regions corresponding to those in Fig. 7. Colloid from this section was also leached out. Where colloid was retained, it was not stained.







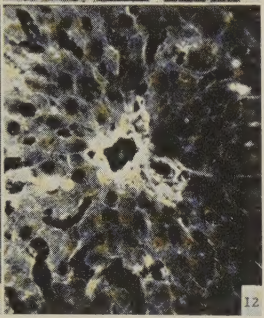


FIG. 9. Lymph node from rat inj. intrav. with anti-kidney serum. Fixation is seen in sub-capsular region as well as in vascular elements. Staining in the vessels is very weak. Capsule and lymphoid cells are unstained.

FIG. 10. Lymph node section treated *in vitro* gives strong staining of the section and is somewhat similar to that seen above, capsule of the spleen, subcapsular region and reticulum of the vessels are fluorescing well. Lymphoids cells are not stained.

FIG. 11. Liver from rat inj. intrav. with anti-kidney serum. Staining is seen in vessels particularly the central veins and in sinusoids radiating from such vascular regions. Sinusoidal staining is very weak and is not apparent in this photograph.

FIG. 12. Liver section treated *in vitro* showing staining of a vessel in the field, stroma surrounding vessel was stained better than *in vivo* section. Sinusoids radiating outward from the vessels are stained, hepatic cells are negative.

directly into contact with intracellular components.

Ovary. (Fig. 1, 2). Strong fluorescence was seen in vascular elements, in stroma surrounding ovarian follicles, and in inner boundary of follicles (region of membrane granulosa and glossy membrane) but the liquor folliculi was negative. In degenerating follicles (Fig. 3), good fluorescence was seen within the follicles. This may be due to the fact that, in the atretic follicle, connective tissue from theca externa penetrates into the lutein mass and forms delicate interlacing septa in which are numerous capillaries which are probably responsible for the observed staining. (Fig. 4 shows *in vitro* staining).

Spleen. (Fig. 5). The capsule of spleen containing elastic fibers and some smooth muscles were unstained. The white pulp stained strongly around the borders, the central artery within the pulp was negative, the red pulp showed some weak areas of staining, the trabeculae were essentially negative. Some macrophages undoubtedly were stained, probably due to phagocytosis of the injected globulin. The other vessels of spleen such as trabecular artery and veins, central artery, pulp artery, and veins, penicillar vessels and venous sinuses were not stained. The strong staining along the periphery of the white pulp may be due to the fact that blood has ready access to this area and reticular fibers and reticular cells are more numerous in this region. (Fig. 6 shows *in vitro* staining).

Thyroid. (Fig. 7). The colloid of the gland did not fluoresce. Neither did the follicular epithelium. Very weak staining was found in the region of reticular connective tissue and capillaries. Due to close proximity of the structures, resolution was difficult. (Fig. 8 shows *in vitro* staining).

Lymph node. (Fig. 9). Specific staining was found under the capsule (subcapsular region), and in elements that appeared to have vascular architecture. Strong staining was seen in polymorphonuclear leucocytes and macrophages. The staining of these and other lymphoid tissue cells may have been nonspecific and due to phagocytosis of the globulin by these cells since similar staining occurred in control sections as well. (Fig. 10 shows *in vitro* staining).

Liver. (Fig. 11) Weak staining was seen in sinusoids surrounding the cords of anastomosing hepatic cells radiating outward from the central veins. The cords themselves were unstained. The strongest intensity was in areas near a central vein. No staining was seen in bile ducts or arteries. The areas which stained are known to be of a reticulum of extremely delicate fibrils, which envelop the sinusoids and a small number of coarser fibers which radiate outward from the region of the central vein. (Fig. 12 shows *in vitro* staining).

Discussion. For an anti-tissue antibody to be cytotoxic for a particular tissue, it must come in contact with the corresponding antigen and be fixed in high enough concentration to cause damage. In the case of injection of anti-rat kidney antisera, there have been many studies showing glomerular damaging effect and there has also been a study indicating malfunction of the adrenal(13). In both of these organs, there have been demonstrated high concentrations of antibody localized on particular structures by means of radiolabelled antibodies(14,15) or by immunohistochemical fluorescein label technics. Localization was found in the glomerular tuft of kidney and the cortical sinusoids of adrenal. In general, immunohistochemical technics have the advantage of greater resolution of the area of fixation if the localized antibody is present at a high local concentration (minimum concentration 28 μg/g), while radiolabel technics are much more sensitive with respect to diffuse localization(9).

We found that anti-RK localizes to high concentration in certain structures of spleen and ovary and to a much lower concentration on structures in thyroid, lymph node and liver; concentrations in brain, testes, skin, heart and lung were too low to be observed.

Physiological effects might be expected on spleen and ovary and perhaps on thyroid, lymph node and liver on the basis of concentration of localized antibody. Apparently these organs possess sufficient amounts of antigen which can come in contact with the circulation and enough circulation to bring adequate amounts of antibody into contact with the structures to permit them to compete successfully for some antibody with those organs, such as kidney, which are known to trap anti-kidney antibodies very efficiently.

It is of interest to note that while every tissue reported here contained antigens capable of reacting with anti-kidney antibodies, localization of antibodies *in vivo* is not apparent. This must be due to the fact that the antibody does not come in contact with the antigen before it is trapped by the more efficient organs because of the route of blood supply or because the antigens are intracellular and not freely available to sequester circulating antibody.

Summary. *In vivo* localization of antibodies prepared in rabbits against rat kidney was determined by immunohistochemical procedures. Fixation to high concentration was observed in certain structures of ovary and spleen as well as in kidney and adrenal. A lower concentration was observed on structures in thyroid, lymph node and liver, whereas none was seen in testes, lung, skin, brain and heart. Localization of these antibodies *in vivo* is quite different from that observed by treating tissue sections *in vitro* with the antibody. In the latter case, many more structures are stained.

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Third Component of Human Complement: Resolution into Two Factors and Demonstration of a New Reaction Intermediate.[*] (25026)

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In presence of Ca^{++} and Mg^{++}, sensitized sheep erythrocytes (EA) react with C′3 deficient human serum (R3) to form a complex ($EAhuC'_A$) which exhibits activities of C′1, C′2 and C′4.[†] If this complex is incubated with serum containing an agent which binds Ca^{++} and Mg^{++}, thus preventing further action of C′1, C′2 and C′4, the cells are hemolyzed by C′3, and the extent of hemolysis can be used as a measure of C′3 concentration(1,2). Evidence has been accumulating, both for human(3) and guinea pig(4,5,6) systems, that more than one factor is involved in this cation independent stage of immune hemolysis. This paper describes the resolution of human C′3 into 2 factors which act sequentially in the lysis of $EAhuC'_A$, and the demonstration of a new reaction intermediate.

Materials and methods. Preparation of EA, R3, $EAhuC'_A$ and ethylenediamine tetraacetate (EDTA) buffer are described in(7). The anion exchanger used, diethylaminoethyl (DEAE) cellulose, is a commercial product containing 0.40 meq/g of ionizing groups on a Solka-Floc lattice. Optical densities were measured with Beckman Model DU spectrophotometer. The fractionation procedure, briefly outlined here, will be published in greater detail elsewhere. A preliminary, low temperature, ethanol precipitation was performed on 20 ml of pooled human serum (pH 5.6, ionic strength 0.1, alcohol 9%, serum 3%). The precipitate was dissolved in 4 ml of 0.08 M phosphate buffer, pH 7.5. Spectrophotometric assay(7) showed that approximately 10-fold enrichment of total C′3 could be achieved in this way with negligible losses. The dialysed fraction was placed on 20 x 1.1 cm DEAE cellulose column of the type developed by Peterson and Sober(8), and subjected to continuous salt concentration gradient elution at constant pH of 7.5(9). The starting buffer was 0.02 M in phosphate and 0.02 M in NaCl. Concentration of NaCl in the eluate was allowed to reach a calculated value(10) of 0.16 M, during which time (3½ hours) twenty 6 ml fractions were collected. The whole operation was carried out in a cold room at 2°C. The eluate fractions were diluted 1 → 20 in EDTA buffer and tested for C′3 by incubation with $EAhuC'_A$. None of them caused lysis. It was suspected that the C′3 had been separated into 2 distinct components, both of which were essential for lysis of $EAhuC'_A$. To test this hypothesis, all eluate fractions were recombined and assayed for C′3 in pairs. The hypothesis was confirmed when one such recombination gave rise to a high degree of lysis. The 2 components have been designated C'_a and C'_b, in accordance with recent publication by Mayer and his group(11) on hemolysis by guinea pig C′.

Results. The first component to emerge from the column, immediately after "break through," has been arbitrarily termed C'_a, and the second, emerging towards the end of elution, C'_b. Optical densities at 541 $m\mu$ of the $EAhuC'_A$ hemolysates resulting from incubation with separate and combined fractions from a typical fractionation are shown in Table I.

After separation of the two C′3 factors, formation of a complex between $EAhuC'_A$ and C'_a, and its subsequent lysis by C'_b, were demonstrated as follows: At 0°C, 50 ml aliquots of a suspension of $EAhuC'_A$ were placed in 3 tubes, A, B and C. After centrifuging at 2°C and pouring off the supernatants, the sedi-

TABLE I. Lytic Activity of Fractions C'_a and C'_b.

Test solution	Optical density of $EAhuC'_A$ hemolysate
2 ml 1 → 10 C'_a + 2 ml EDTA buffer	.047
2 ml 1 → 10 C'_b + 2 ml EDTA buffer	.049
4 ml EDTA buffer	.053
2 ml 1 → 10 C'_a + 2 ml 1 → 10 C'_b	1.19

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[*] Supported in part by Grant from Nat. Inst. of Health.

[†] C′1, C′2, C′3 and C′4 represent the 4 recognized components of complement.

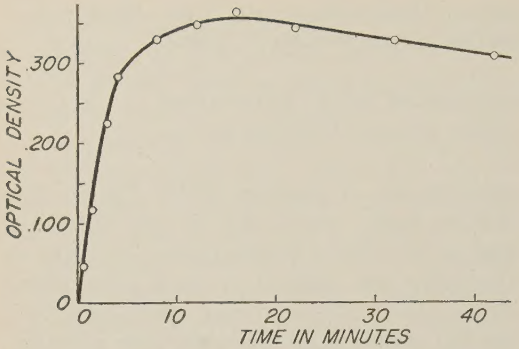
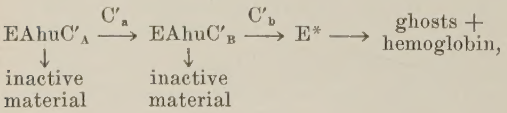


FIG. 1. Formation and decay of intermediate complex EAhuC'_B by action of C'_a on EAhuC'_A.

mented cells were resuspended by tapping tubes with the fingers. Tubes were then placed in 32°C water bath and immediately 15 ml of a 1 → 30 dilution of C'_a in EDTA buffer added to A and B, and 15 ml of buffer to C. Mechanical agitation of reaction vessels was maintained during experiment. The C'_a and buffer had been equilibrated at 32°C. Samples (1.5 ml) were removed at intervals and delivered into 9 ml of ice cold EDTA buffer to stop further reaction. The diluted suspensions were centrifuged in the cold within 2 minutes of sampling. After sampling and centrifugation were completed, each lot of sedimented complex from A and C was treated with 3 ml of 1 → 20 dilution of C'_b in EDTA buffer and incubated 1 hour at 32°C. Those from B were incubated at 32°C for one hour with EDTA buffer alone. No samples from control vessels B and C gave any lysis above that of a "cell blank" (EAhuC'_A + EDTA buffer, 1 hour at 32°C – optical density of hemolysate, 0.042). The samples from vessel A, on the other hand, gave lysis which increased rapidly with time of sampling until a maximum was reached, then slowly decreased. The lysate optical densities, corrected for "cell blank," are plotted against sampling time in Fig. 1.

Discussion. The evidence presented indicates that terminal stages of immune hemolysis in the human system may now be formulated:



$$\begin{tikzcd}
\text{EAhu}C'_A \arrow[r, "C'_a"] \arrow[d] & \text{EAhu}C'_B \arrow[r, "C'_b"] \arrow[d] & E^* \arrow[r] & \text{ghosts + hemoglobin,} \\
\text{inactive material} & \text{inactive material} & &
\end{tikzcd}$$

where EAhuC'_B represents a new reaction intermediate, and E* a cell in a damaged state (12). These final stages, unlike conversion of EA to EAhuC'_A by C'1, C'2 and C'4, require no Ca^{++} and Mg^{++}, and can thus take place in presence of EDTA. Decay of EAhuC'_A to inactive material is known to occur rapidly at 32°C(1) and, unless massive concentrations of C'_a are present, only a relatively small proportion is likely to be converted to EAhuC'_B. Decay of EAhuC'_B, on the other hand, is comparatively slow (Fig. 1), and it seems probable that most of the complex is converted to E* by C'_b under normal conditions of hemolysis.

Solutions of C'_b have been found to retain their activity over periods of several weeks if kept at –70°C. Solutions of C'_a are less stable, but decomposition may be delayed by addition of gelatin to final concentration of 0.05%. Separation of C'3 into 2 factors and demonstration of the intermediate EAhuC'_B promises to simplify greatly the task of investigating the mechanism of terminal stages of hemolysis.

Summary. Human C'3 has been resolved into 2 factors, C'_a and C'_b. These factors acted in the order C'_a, C'_b, in the conversion of EAhuC'_A to E*. Formation and decay of a new intermediate complex between sensitized erythrocytes and human complement, EAhuC'_B has been demonstrated.

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